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The Journal of Biochemistry, Vol. 46, No. 3, 1959

A NEW HEMATIN COMPOUND ISOLATED FROM EUGLENA GRACILIS

By MITSUO NISHIMURA*

(From the Botanical Institute, Faculty of Science, University of Tokyo, Tokyo and the Tokugawa Institute for Biological Research, Tokyo)

(Received for publication, July 7, 1958)

Euglena gracilis has a property of losing chlorophylls when cultured heterotrophically in the dark. When the colorless cells thus produced are grown photo-autotrophically under illumination, they synthesize gradually chlorophylls *a* and *b* with the development of photosynthetic activity (*1*, *2*). During the investigation on this phenomenon, we found that in the process of greening of the dark-cultured white cells, there occurred, concomitant with the appearance of chlorophylls, a formation of a cytochrome which had been undetectable in the original colorless cells. It is the purpose of this paper to describe the method of isolation and some properties of this cytochrome which differs in some significant points from other cytochromes thus far reported in the literature. Tentatively this cytochrome may be designated by the name *Euglena*-cytochrome-552.

MATERIAL

Euglena gracilis var. *bacillaris* was cultured for five to seven days at 25° in a modified medium of Cramer and Myers, containing butyrate and glutamate as carbon sources (*1–3*). The green cells were cultured in an incubator illuminated with daylight fluorescent lamps; the light intensity was 6,000–10,000 lux. The green cells contain chlorophylls *a* and *b* and two kinds of carotenoids. When the white cells were cultured successively in the dark, chlorophylls *a* and *b* were completely lost, whereas protochlorophyll was detected in trace (*1*, *2*). The two kinds of carotenoids were also found, but less in quantity than in the green cells. The loss of chlorophylls also occurred in the mutants obtained by streptomycin treatment or by heat treatment (at 38°). Unlike the colorless cells obtained by dark-cultures these cells were found to be unable to form chlorophylls both in the light and in the dark.

RESULTS

Preparation of Euglena-cytochrome-552—The green cells of *Euglena* were harvested by centrifugation, and washed twice with 0.5 per cent NaCl solution. To 20 g. of fresh *Euglena* cells 10 ml. of 0.2 *N* H_2SO_4 were added, and the cells were ground at 0° in a mortar with quartz sand. The homogente thus obtained was kept at room temperature for 60 minutes, and centrifuged (2×

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$10^4 \times g$, 15 minutes) in a refrigerated centrifuge. The green precipitate was discarded and the reddish brown supernatant fluid, pH 3.8–4.0, was neutralized to pH 7.0 with NaOH. To the neutralized solution ammonium sulfate was added to make a 0.45 saturated solution, and kept in a refrigerator overnight. White precipitate formed was centrifuged off, and the red supernatant solution was made to 0.9 saturation by addition of ammonium sulfate, and kept in a refrigerator overnight. The precipitate formed was collected by centrifugation and dissolved in pure water. With this solution the ammonium sulfate fractionation was repeated again; the red precipitate formed was dissolved in a minimum volume of water, and dialyzed in a cellophane sack against pure water. A little turbidity produced was removed by centrifugation and the solution of cytochrome was obtained. Further purification could not be performed owing to the insufficiency of the starting material.

Properties of Euglena-cytochrome-552—By paper electrophoresis it was revealed that the solution obtained by the above procedure contained some colorless proteins but only one red component which moved toward the cathode, the cathodic mobility at pH 6.0 in *M*/20 phosphate buffer being about two-thirds of that of horse heart cytochrome c.

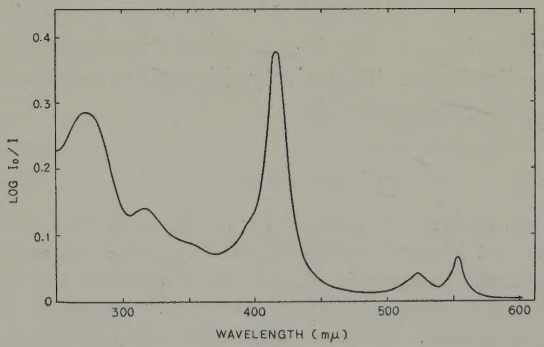

FIG. 1. Absorption spectrum of reduced *Euglena*-cytochrome-552 (reduced with hydrogen and platinum-asbestos).

The solution of the red substance showed a characteristic absorption spectrum of cytochrome c group; the absorption maxima in the reduced state (reduced with hydrogen and platinum-asbestos) were at 275 mμ, 317 mμ, 417 mμ, 523 mμ and 552.5 mμ, with a shoulder at 355 mμ (Fig. 1) and the absorption maxima in the oxidized state (after oxidation with potassium ferricyanide according to Davenport and Hill (*4*)) were situated at 275 mμ, 355–360 mμ, 412 mμ and 530 mμ (Fig. 2). The substance was non-autoxidizable in air and did not combine with carbon monoxide.

The absorption maximum in the ultraviolet region, 275 mμ, in the reduced and oxidized forms, was shifted to 268–270 mμ after precipitation with acetone, a fact showing the possibility of partial denaturation caused by the acetone treatment. The reduced pyridine hemochromogen prepared

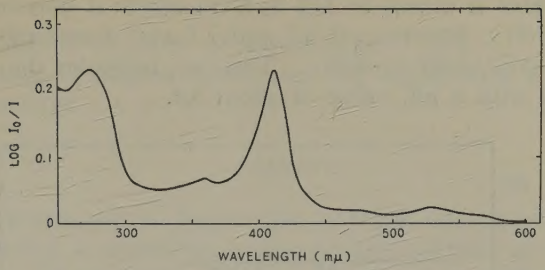

FIG. 2. Absorption spectrum of oxidized *Euglena*-cytochrome-552 (oxidized with potassium ferricyanide; see text).

from the substance showed an absorption maxima at 415 mμ, 523 mμ, and 550 mμ, indicating that it contained a heme that was identical with or similar to that of the mammalian cytochrome c.

Oxidation-Reduction Potential—Measurements of oxidation-reduction potential were carried out according to the method of Davenport and Hill (*4*), using a redox buffer solution of potassium ferricyanide-ferrocynide. E_0' of the ferricyanide-ferrocyanide system at 25° was assumed to be 0.409 v., calculated from the data of Schaum and Linde (*5*). The measurements were carried out anaerobically at 25±1°.

Some examples of the measurements are shown in Fig. 3. A plot of the logarithms of the ratio of ferrocytochrome to ferricytochrome against the logarithms of ferrocyanide/ferricyanide gave a straight line with a slope of 45°, indicating that one electron change was involved in the oxidation-reduc-

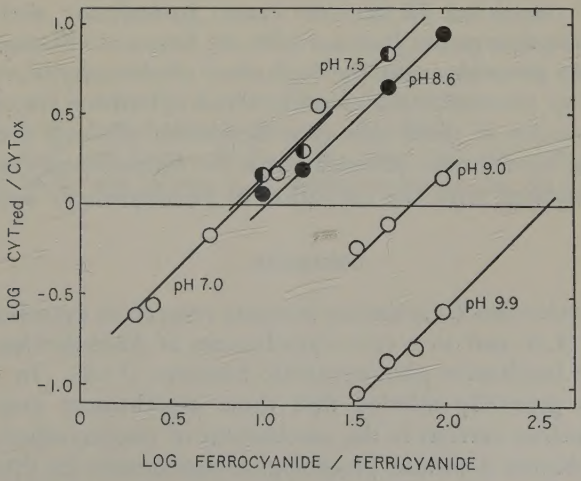

FIG. 3. Effect of ratios of ferrocyanide to ferricyanide on the proportion of reduced and oxidized cytochromes.

tion of the cytochrome.

From these data E_0' of the cytochrome was calculated. The effect of

pH on the E_0' value is shown in Fig. 4, from which it may be seen that E_0' was constant at 0.36 v. between pH 6.2 and 7.7 and decreased in higher pH ranges at a rate $\Delta E_0'/\Delta pH = -0.06$ v. This fact indicates the presence of a dissociable group with a pK value of about 8.3.

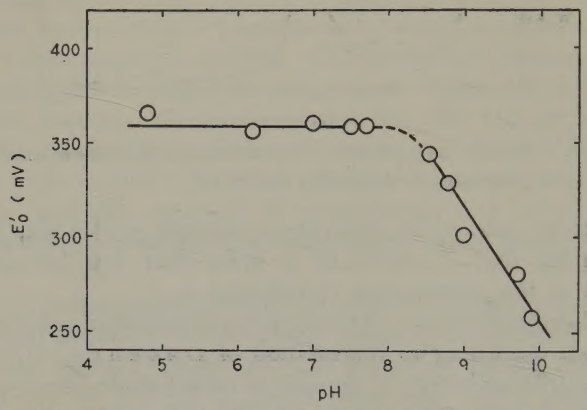

FIG. 4. Effect of pH on the E_0' of *Euglena*-cytochrome-552.

Association of Euglena-cytochrome-552 with Photosynthetic Apparatus—The cytochrome having the properties described above could be extracted from the green *Euglena* cells cultured in the light, but not from the dark-grown white cells, nor from the colorless mutants obtained by streptomycin- or high temperature-treatment.

The dark-grown white cells were suspended aseptically in *M*/50 phosphate buffer (pH 6.8) containing 0.2 per cent casein hydrolyzate and illuminated at 8,000 lux with fluorescent lamps. After 46 hours of illumination at 25°, the cells became greenish (with the formation of chlorphylls *a* and *b*) and began to develop photosynthetic activity without, however, increasing in cell number*. Extraction of these cells gave a solution showing a characteristic spectrum of the cytochrome, indicating that the formation of the cytochrome was closely associated with the formation of photosynthetic apparatus.

DISCUSSION

Euglena-cytochrome-552 is similar in many respects to cytochrome f found in green plants (*4*, *6*) and to c type cytochromes of *Rhodospirillum* and *Rhodopseudomonas*, the facultative photosynthetic bacteria (*7*~*9*). In view of the possibility now generally alluded that these cytochromes may be playing some role as electron carriers in the mechanism of photosynthesis, it may be reasonable to assume a similar physiological significance for this cytochrome in the green *Euglena*-cells.

With a species of *Euglena* Davenport and Hill (*4*) reported the existence of a cytochrome(s) showing a broad band which they conjectured

* *Euglena* cells do not multiply in the medium used in this experiment (*1*).

to be a mixture of α-bands of cytochromes f and c. Presumably they observed the band of *Euglena*-cytochrome-552 mixed with a band(s) of some other cytochrome(s).

SUMMARY

From the green cells of *Euglena*, the isolation was carried out for a new cytochrome having a prosthetic group similar to that of the mammalian cytochrome c. Characteristic of this cytochrome are its high oxidation-reduction potential ($E_0' = 0.36$ v.) and its close association with photosynthetic apparatus. The chlorophyll-less cells produced either by prolonged heterotrophic dark culture or by mutation induced by streptomycin- or heat-treatment contain no trace of the cytochrome. The new cytochrome, which seems to play some role in the mechanism of photosynthesis of the green *Euglena*, was tentatively named *Euglena*-cytochrome-552.

The author expresses many thanks to Prof. H. Tamiya and Prof. A. Takamiya for their kind guidance and valuable criticism. He is also indebted to Prof. H. Huzisige for his suggestions. This work was supported in part by a research grant from the Ministry of Education.

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The Journal of Biochemistry, Vol. 46, No. 3, 1959

STUDIES ON THE CHLOROPHYLL FORMATION IN EUGLENA GRACILIS WITH SPECIAL REFERENCE TO THE ACTION SPECTRUM OF THE PROCESS

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(Received for publication, July 7, 1958)

The light-induced synthesis of chlorophylls as well as the appearance of photosynthetic activity in the "etiolated" cells of *Euglena* has been a subject of investigation in this laboratory. In one of the previous studies of the series (*1*), we followed the course of chlorophyll synthesis as influenced by various environmental factors, in particular, the presence and the concentration of nitrogenous substances and streptomycin added to the incubation medium. In the present paper, some results of further studies will be reported, in which the influence of light factors was investigated and action spectrum for the process of chlorophyll formation was obtained. The absorption spectrum of the etiolated cells was also studied. From the results of these experiments protochlorophyll was found to be functioning as the actual precursor in the chlorophyll formation of *Euglena*.

METHODS

Organism—The organism used in this study was the same strain of *Euglena gracilis* var. *bacillaris* as that used in the previous work (*1*). The cells were freed of chlorophyll pigments by repeated subculturing in the dark (*white cells*). The cultures were made on a medium of the following composition (modified Cramer and Myers' medium (*1, 2*));

Butyric acid	2.0 ml.	$CoCl_2 \cdot 6H_2O$	1.1 mg.
NaH glutamate$\cdot H_2O$	1.0 g.	$ZnSO_4 \cdot 7H_2O$	0.4 mg.
$(NH_4)_2HPO_4$	0.5 g.	$(NH_4)_2MoO_4$	0.2 mg.
KH_2PO_4	0.5 g.	$CuSO_4 \cdot 5H_2O$	0.02 mg.
$MgSO_4 \cdot 7H_2O$	0.2 g.	Thiamine hydrochloride	0.1 mg.
Na_3 citrate$\cdot 2H_2O$	0.2 g.	Vitamine B_{12}	0.1 μg.
$CaCl_2$	20.0 mg.	Water to	1000 ml.
$Fe_2(SO_4)_3$	2.4 mg.	(pH adjusted to 6.8 with NaOH)	
$MnCl_2$	1.1 mg.		

Three liters of the culture medium were inoculated with 20 ml. of a culture of the dark-grown *white cells* of *Euglena* (0.5 mg. dry weight/ml.) at the logarithmic stage of growth. After being incubated for 5 days in the dark at 25°, when the organism was in the phase of

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logarithmic growth, the cells were aseptically harvested and washed with 0.5 per cent NaCl solution by centrifuging in the dark. Usually a yield of 0.75 g. dry weight of the white cells per bottle was obtained.

The normal, *green cells* of *Euglena*, for the purpose of control, were obtained by growing the organism in the same medium, but under continuous illumination.

Optical Equipment—Fig. 1 illustrates the general plan of the equipment used. A 1,000 w. tungsten incandescent lamp operated at 100 v. A. C. was the light source. Monochromatic lights of good purity were obtained with varied combinations of glass absorption filters (Baird Associates, Inc. and Tokyo Shibaura Electric Co.) together with interference filters (Baird Associates, Inc.). The wavelengths of maximal transmission and the transmission curves for each set of filters are presented in Table I and Fig. 2, respectively. The

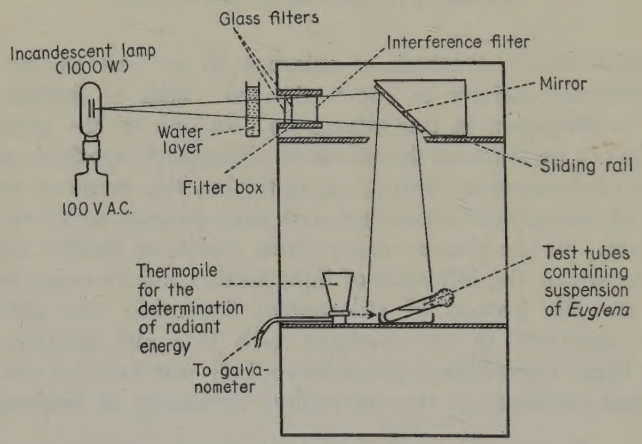

FIG. 1. General plan of the optical equipment.

TABLE I

Combinations of Filters

No.	Combinations of Baird interference filters and Baird glass filters	Toshiba glass filters	Transmission maxima (mμ)
1	V-412-136	V-B3A-1	456
2	V-462-151	V-Y1A-1	512
3	V-358-122	V-G1A-2	536
4	V-316-123	V-G1A-1	554
5	7-5189-6	IRO-1B-2	597
6	7-5177-1	IRO-1B-1	645
7	V-51	IRO-1B-2	700
8		V-V2-1 V-V2-2	395

transmittance was measured with Shimadzu Model QB-50 Electric Spectrophotometer. The each set of filters was mounted on a wooden box arranged around the incandescent lamp used as the common light source. A layer of water (5 cm. thick) was placed between the lamp and the filters. The light, introduced horizontally into the box through the

sheets of filters, was reflected with a glass mirror at an angle of 45° to give a vertical illumination of the flagellate suspension in the test tube. The energy of the light was measured with a large-surface Kipp thermopile, Type E1, with a glass window, the instrument being inserted at the exact position at which the test tube containing the organisms was to be illuminated. The galvanometer readings with the thermopile were calibrated against a carbon filament standard lamp (U. S. Bureau of Standards). The distance of each box from the incandescent lamp was adjusted so as to give the same intensity of radiant energy of 1.39×10^3 ergs $cm.^{-2}$ $sec.^{-1}$ at the site of illumination. The whole set of boxes was placed in a dark room maintained at a temperature of 26.5°.

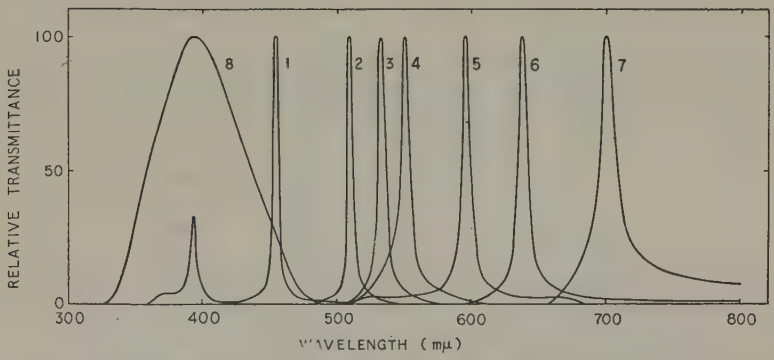

FIG. 2. Transmission curves obtained with combinations of filters listed in Table I.

Illumination of the Organism—The washed cells of dark-grown *Euglena* were resuspended a in phosphate buffer (pH 6.8; *M*/50) with an addition of vitamin-free casein hydrolyzate in a final concentration of 2 mg./ml. The density of the *Euglena* cells in the suspension amounted to 2.3 mg. dry weight/ml. Aliquots of 11 ml. of the cell suspension were pipetted into cotton-plugged test tubes, which were then illuminated in the above-described set of the boxes with the monochromatic lights of the given wavelengths. All the above procedures were performed aseptically. The tubes removed at regular intervals of illumination were heated for 2 minutes at 90°, and the determinations of chlorophyll formed were performed for 10 ml. portions of the heated suspension in the test tubes.

Determination of Chlorophylls—Pigments were extracted with acetone from 10 ml. of the heated suspension (see above). The concentrations of chlorophylls *a* and *b* were determined by measuring the optical density of the 80 per cent acetone extract at 645 mμ and 665 mμ; calculations were made using the values for the specific absorption coefficients of chlorophylls *a* and *b* reported by Mackinney (*3*).

Absorption Spectra of Euglena Cells and Barley Leaves—The washed *white cells* of *Euglena* were killed by heating the suspension for 2 minutes at 90°. The cells were then suspended in a 40 per cent sucrose solution (dry weight of *Euglena* cells in suspension: 0.82 mg./ml.), and their absorption spectrum was determined by the opal glass method of Shibata *et al.* (*4*), using oiled filter paper as the light-scattering plate. The length of the optical path in the measurement was 1 cm.

Barley seedlings were grown in the dark at 25°. The etiolated leaves thus obtained were heated for 2 minutes at 90°; all procedures being carried out in complete darkness. The absorption spectrum was measured as described above.

RESULTS

Chlorophyll Content of Green Cells and White Cells of Euglena—The levels of chlorophylls found in the *green* and *white* cells of *Euglena* were as follows:

Green cells	chlorophyll *a*	$2.5-3.2\times10^{-2}$ mg./mg. dry weight
	chlorophyll *b*	$5.0-6.6\times10^{-3}$
White cells	chlorophylls *a* and *b*	nil
	protochlorophyll	$7.2-17.8\times10^{-6}$

Protochlorophyll in the normal green cells could not be successfully determined, because of its exceedingly low concentration as compared to those of chlorophylls *a* and *b*.

Changes in Chlorophyll Pigments during Illumination of White Cells—Changes that occurred upon illumination of the resting *white cells* of *Euglena* are illustrated in Fig. 3. A markedly rapid decrease in protochlorophyll, compensated by

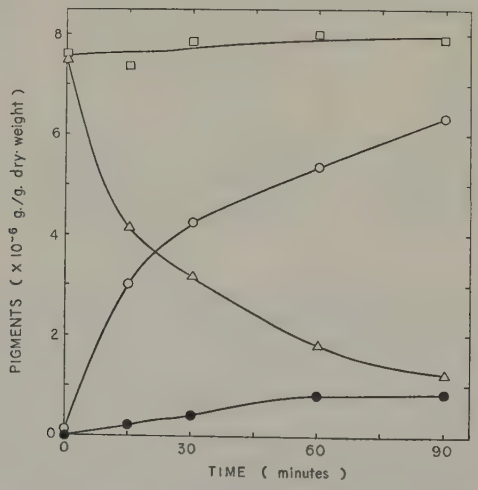

FIG. 3. Changes in chlorophylls in the earlier stage of illumination. —△— protochlorophyll, —○— chlorophyll *a*, —●— chlorophyll *b*, —□— protochlorophyll+chlorophylls *a* and *b*.

a no less prominent increase in chlorophyll *a* will be noticed. The level of chlorophyll *b* also showed a rise, which, however, was not very significant. The total amounts of these three components of chlorophyll pigments in the cell were found to increase by only an insignificant extent during the period of the illumination in this experiment. The same results, when replotted on a semi-logarithmic scale (Fig. 4), revealed, at least for the initial part of the change, a first order reaction with respect to the concentration of protochlorophyll in the cell. Calculating from the results shown in Figs. 3 and 4, a half-life time of about 24 minutes was obtained for the disappearance of protochlorophyll under the conditions of the present experiment. The

time course of formation of chlorophyll *a* was also found to be roughly in accord with the first order reaction of the conversion of protochlorophyll into chlorophyll *a*:

$$\text{Protochlorophyll} \xrightarrow{\text{light}} \text{Chlorophyll } a.$$

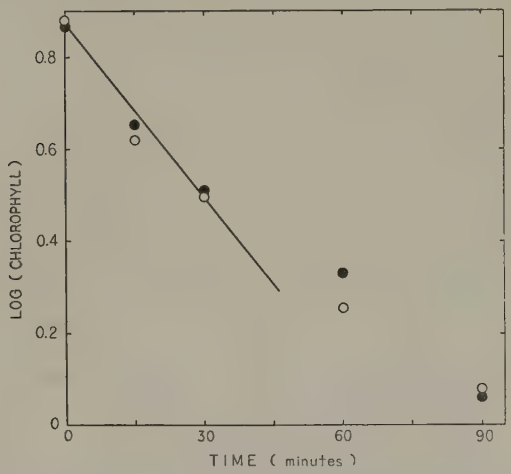

FIG. 4. Transformation of protochlorophyll to chlorophyll *a*. —○— log (protochlorophyll), —●— log (protochlorophyll (at 0 minute)—chlorophyll *a*).

In the above experiment, the *white cells* of *Euglena* were suspended in a *M*/50 phosphate buffer (pH 6.8). Aliquots of 100 ml. were illuminated in flat Roux flasks (thickness of layer of the suspension, 6.8 mm.) with the white light from a tungsten incandescent lamp, passed through an appropriate layer of water to remove heat; the intensity of the illumination in this case was 8,800 lux; the concentration of the cell suspension, 18.1 mg. dry weight/ml. The concentrations of protochlorophyll, chlorophylls *a* and *b* were determined by the method of K o s k i (*5*).

Increase of Chlorophyll Content in Illuminated White Cells—The above-stated changes involved in the initial stage of the illumination consisted mainly in the conversion of the precursor of chlorophyll into chlorophyll. The total amount of the precursor, most probably protochlorophyll, however, was as low as about 1×10^{-5} g. per g. dry weight of white cells. After almost complete conversion of this amount of protochlorophyll into chlorophyll(s), the appearance of the test material remained nevertheless almost unaltered, the level of the green pigment thus attained being as low as 1/3000 of that ordinary contained in the green *Euglena* cells. Actual increase in chlorophyll content did not immediately succeed the above stated step of protochlorophyll transformation. A lag period of about 15 hours was always noticed before the steady rise in the chlorophyll level actually began (see also *ref.*(*1*)). During this lag period and also afterwards, in

the later stage of chlorophyll synthesis, no appreciable amount of protochlorophyll was ever discovered in the cells. This finding is suggestive of the fact that the over-all rate of chlorophyll synthesis under investigation is determined by the rate of formation of protochlorophyll in the cell, since its conversion into chlorophyll, as we have studied above, is a fairly rapid process in itself. The effects of various environmental factors on the process of chlorophyll synthesis have been the subject of our previous study. In the present work, the effect of the illumination was investigated more quantitatively and with the monochromatic lights.

Action Spectrum of Chlorophyll Formation—The rate of chlorophyll synthesis (steady phase) in the *white cells* of *Euglena* was found to be dependent on the wavelength as well as the intensity of the monochromatic light with which the cells were illuminated.

The time course of chlorophyll formation in the *white cells* illuminated with the monochromatic lights is shown in Fig. 5. The rate of chlorophyll

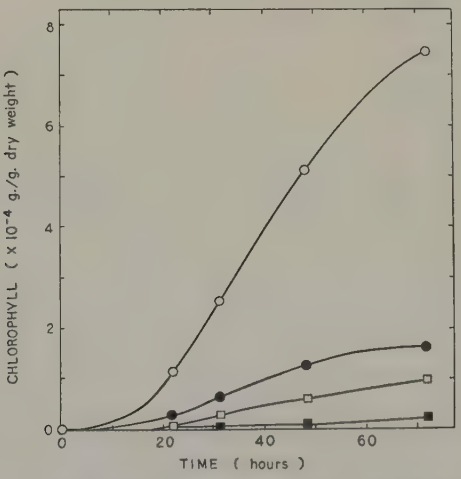

FIG. 5. Time course of the chlorophyll formation. —○—, chlorophyll *a*, filter combination No. 8 (main wavelength 395 mμ); —●—, chlorophyll *b*, filter combination No. 8; —□—, chlorophyll *a*, filter combination No. 3 (main wavelength 536 mμ); —■—, chlorophyll *b*, filter combination No. 3.

formation is expressed by the amounts of chlorophylls *a* and *b* formed during the period 21.75–72 hours of the illumination, which are plotted in Fig. 6 against the wavelength of the light applied. The intensity of the monochromatic light used in the present experiments was by far lower than the level of light saturation, so that even the highest yield in chlorophyll formed was by certain orders of magnitude less than that obtainable under sufficient illumination (*1*). It may, therefore, be justificable to conclude that the results thus obtained (Fig. 6) gave the actual action spectrum of the chlorophyll synthesis in question. The general feature of the action spectra

obtained was similar to the absorption spectrum of chlorophyll, showing a common maximum at about 650 mμ, a minimum at about 500 to 550 mμ and a steep rise towards shorter wavelengths. No marked rise in the curves

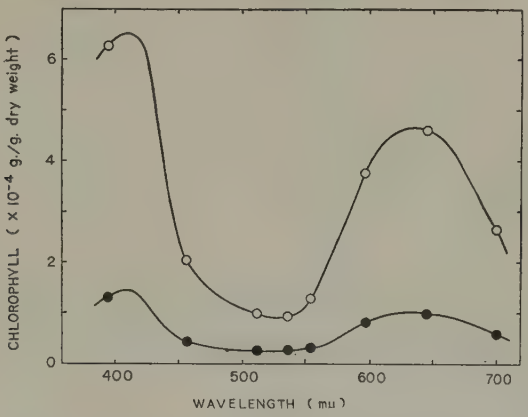

FIG. 6. Action spectra of chlorophyll formation.
—○— for chlorophyll *a*, —●— for chlorophyll *b*.

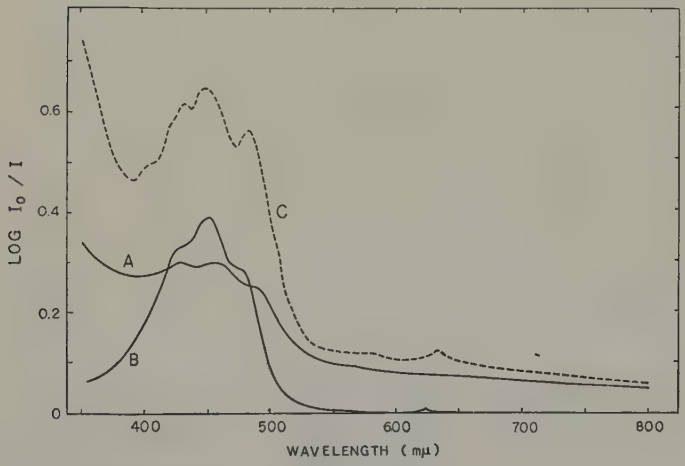

FIG. 7. Absorption spectra of *Euglena* and etiolated barley leaves.
A: absorption spectrum of the suspension of the cells of *Euglena* cultured in the dark (0.82 mg./ml.). B: absorption spectrum of ether solution of the pigments extracted by 80 per cent acetone from dark-cultured *Euglena* (4.6 mg. *Euglena* (dry weight)/ml. ether). C: absorption curve of etiolated barley leaves.

was discovered in the range from 450 mμ to 500 mμ, around which the maxima of absorption of carotenoid pigments lie.

Absorption Spectra of Euglena Cells—The absorption spectrum of a suspension of the (heat-treated) *white cells* of *Euglena* is presented in Fig. 7, Curve A. It

showed two peaks, at 430 mμ and 457 mμ, and a shoulder at 490 mμ; absorption due to protochlorophyll was not observed in this case. Curve B is the absorption curve obtained with an ether solution to which the pigments were transferred from an 80 per cent acetone extract of the white *Euglena* cells. It showed an absorption maximum at 451.5 mμ with two shoulders, at 420 mμ and at 480 mμ, besides a very low but distinct peak at 623 mμ. The absorption at 623 mμ is due to the content of protochlorophyll, as is more evidently shown in Fig. 8, which gives the absorption spectrum of protochlorophyll extracted from the *white cells* of *Euglena* and purified by paper chromatography. Because of weakness of the absorption band involved, the presence of protochlorophyll could not be demonstrated directly with the suspension of the *white cells* (Fig. 7, Curve A.) In the case of the etiolated barley leaves, the absorption band of protochlorophyll was successfully demonstrated, using the heat-treated leaves (Fig. 7, Curve C).

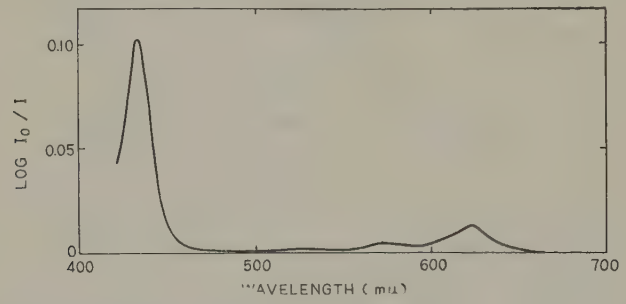

FIG. 8. Absorption spectrum of protochlorophyll extracted from *white cells* of *Euglena* (dissolved in ether).

DISCUSSION

From the results of the present study, it will be readily concluded that protochlorophyll was involved as the precursor in chlorophyll synthesis in the *white cells* of *Euglena* (see Fig. 3). The conversion of the bulk of the initial content of protochlorophyll in the cell into chlorophylls constitutes the first stage of chlorophyll synthesis under investigation.

Although we have had as yet no definite proof as to the sequence of formation of the two components *a* and *b* of chlorophyll, the earlier appearance of the former component will be taken as evidence for the fact that the *b* component is formed, as is known to be usual with green leaves, by the subsequent change of the pre-formed chlorophyll *a*, to give rise to a stationary ratio of 0.20–0.22 of chlorophyll *b* to chlorophyll *a*.

Protochlorophyll- - - - - - - - →Chlorophyll *a*

└- - - - - - →Chlorophyll *b*

Because of the technical difficulties involved, we could not obtain the action spectrum for this first stage of chlorophyll formation.

The time lag, always observed before the further steady increase in the chlorophyll level took place in the *white cells* under illumination, is indicative of the circumstance that certain chemical processes were required to initiate further steady production of protochlorophyll in the cell. The duration of this lag was not influenced by varying the intensity of the wavelength of the illumination. The addition of several nitrogenous substances such as casein hydrolyzate to the reaction mixture has been found to be effective in curtailing the lag phase (*1*). These findings lead us to assume that the preparatory processes in question belong to the class of dark reactions. Once the precursor has been produced, its rapid conversion into chlorophyll will follow, to give rise to the actual increase in the chlorophyll content of the cell.

The above-described action spectrum indicates that chlorophyll plays a role as the principal photosensitizing agent(s) in this steady stage of chlorophyll formation. Since a limited number of the isolated monochromatic lights was used in this study, we could not obtain action spectra that would furnish sufficient details for deciding which component was actually functioning as the main photoreceptor. The generally accepted view that protochlorophyll is playing the part of the main sensitizing agent in the chlorophyll synthesis may perhaps be applicable here, so far as the initialphase of chlorophyll synthesis in the *white cells* is concerned, the process consisting in the conversion of the "reserve" protochlorophyll into chlorophyll (*a* and *b*), and there being no significant amount of other photoreactive pigments whatsoever detectable in the *white cells*. In later stages of pigment formation, however, the cells become more and more enriched in newly formed chlorophyll, the level of which soon overruns that of the precursor, protochlorophyll, as we have described above. Therefore, there is a probability that chlorophyll(s) effectively takes part in transferring light energy for the synthetic process under investigation. Participation of carotenoids as the principal photosensitizers was excluded by the finding that the action spectrum showed a maximum in the region of wavelengths where carotenoids are known to exhibit no significant absorption.

Generally speaking, our experimental results are similar to those reported by Wolken *et al.* (*6*, *7*) who worked with the proliferating cells of *Euglena*. It ought to be noticed, however, that their starting material (*i.e.*, cells grown in complete darkness for 7 to 10 days before experimentation) still maintained a considerable level of chlorophyll content, so that only an increase by several fold was achieved after 96 hours of incubation in the light. Moreover, they have noticed that under the condition of their experiments their test organism showed a more or less significant loss in chlorophyll when kept in the dark. The circumstances seem to be more favorable for investigation in our case, in that the synthesis was followed starting from zero level of pigmentation and that there was no tendency for the gradual decrease in the chlorophyll level when the organism was incubated in total darkness for a period as long as 20 hours in non-proliferating state.

SUMMARY

1. The *Euglena* cells synthesize chlorophylls *a* and *b* in the light. In the dark, chlorophylls are not formed, and protochlorophyll is detected in a very small amount. The protochlorophyll present in the white cells was extracted and identified.

2. The photochemical transformation of protochlorophyll to chlorophyll *a* was shown to occur in the dark-grown cells in the initial stage of illumination.

3. After the illumination was started, the actual increase in chlorophyll content occurred following a lag period of about 15 hours.

4. The rate of chlorophyll formation in the non-proliferating suspensions of *Euglena* depends on the wavelength of the illumination. The action spectrum of chlorophyll formation has two peaks; one lies about 650 mμ, the other in the violet region of the spectrum. Chlorophyll is inferred to be the principal photosensitizer in the later period of chlorophyll formation.

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ACTION OF CARBOXYPEPTIDASE ON SYNTHETIC SUBSTRATES

I. ACTION OF CARBOXYPEPTIDASE ON MONO-, DI-, TRI-, TETRA- AND PENTA-GLYCYL-L-TYROSINE*,**

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The rates of hydrolysis of synthetic substrates by CPase depend chiefly on the nature of the C-terminal residue, and acyl dipeptide analogs containing aromatic amino acid and leucine of the L-configuration in the C-terminal position fulfil optimal structural requirements; CbzGly-L-Phe is most sensitive among substrates known for the enzyme (*1*).

Although the side chain specificity of the amino acids forming the susceptible bond is a dominant factor, it should be mentioned that the rate of release of C-terminal amino acid from a protein may also be influenced by the environmental conditions imposed on the sensitive bond by virtue of the size and spacial configuration of polypeptide chains in the molecule. In this connection, it seemed desirable to examine the mode and rates of hydrolysis of the peptides Gly_n-L-Tyr by CPase in which n are 1, 2, 3, 4 and 5. Moreover, a study designed in this paper appeared valuable in view of the widespread use of the enzyme to determine C-terminal residues and amino acid sequences of proteins and peptides (*2*).

Earlier studies have shown that CPase hydrolyzes, though at low rates, the dipeptides such as Gly-L-Phe (*3*), Ala-L-Phe (*3*), Gly-L-Tyr (*4*, *5*), Gly-L-Try (*5*), Gly-L-Leu (*5*), L-Tyr-L-Tyr (*4*) and Leu-L-Tyr (*5*). However, the introduction of glycyl residue to N-terminal position of L-Leu-L-Tyr resulted in remarkable increase in hydrolytic rate; Gly-L-Leu-L-Tyr was hydrolyzed 38 times more rapidly than L-Leu-L-Tyr (*3*). Plentl and Page have shown that the terminal peptide bond of the tetrapeptide L-Tyr-L-Lys-L-Glu-L-Tyr was susceptible to hydrolysis by CPase (*6*).

Yanari and Mitz have shown that L-LeuGlyGly and L-LeuGly are resistant to hydrolysis even with relatively large amount of CPase (*5*). In

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** The following abbreviations are used: CPase, pancreatic carboxypeptidase; Tris, tris(hydroxymethyl)-aminomethane; Cbz, carbobenzoxy; OBz, benzyl ester; DMF, dimethylformamide. Abbreviations for the amino acid residues are those suggested by Brand and Edsall, *Ann. Rev. Biochem.*, **16**, 224 (1947).

the present investigation, it was shown by means of paper chromatography that the peptides Gly_n-L-Tyr (n=2∼5) are hydrolyzed to corresponding Gly_n (n=2∼5) and L-tyrosine by CPase, the polyglycine Gly_n (n=2∼5) being not split appreciably further.

In the synthesis of the substrates tested, the mixed anhydrides of $CbzGly_n$ (n=1∼4) were coupled with L-tyrosine benzyl ester or glycyl-L-tyrosine benzyl ester by the procedure of Vaughan and Osato (*7*), and after hydrogenation of the coupling products, the peptides Gly_n-L-Tyr (n=1∼5) were obtained.

EXPERIMENTAL

Enzyme

A crystalline CPase from Worthington Biochemical Corp., N.J., U.S.A. (3 times recrystallized; CO 570) kindly supplied by Dr. J.P. Greenstein was used throughout the experiments. Enzyme solutions in the presence of 2 *M* LiCl were made up daily from a stock solution containing about 0.05 mg. of protein N per ml. The stock solution in 2 *M* LiCl was prepared about every 4th day from a stock suspension of crystals. Protein N concentrations were determined by measurements in Shimadzu spectrophotometer (QB-50) from a standard calibration curve at 280 mμ, which was established by protein N analysis by Kjeldahl method.

The purity of enzyme was established by its activity towards CbzGly-L-Phe and CbzGly-L-Tyr. At 25° in 0.1 *M* LiCl and 0.05 *M* phosphate buffer at pH 7.5, the enzyme hydrolyzed 0.05 *M* CbzGly-L-Phe with a proteolytic coefficient of 13.7. Values of 13 (25°, 1/30 *M* phosphate at pH 7.3) (*8*) and 13∼14 (25°, 0.1 *M* LiCl and 0.04 *M* phosphate at pH 7.5) (*9*) have been recorded in the literatures. Similarly, the enzyme attacked 0.05 *M* CbzGly-L-Tyr with a proteolytic coefficient of 7.8. A value of 6.2 has been reported (*8*).

Methods

Unless otherwise stated, assays of enzyme activity were performed by the following procedure. 0.2 ml. of enzyme solution in 2 *M* LiCl was added to a 2 ml. flask containing a substrate in a specified concentration buffered with 0.2 *M* Tris at pH 8.5. Solution was made up to 2 ml. by water, the final concentrations of Tris buffer and LiCl being 0.05 *M* and 0.1 *M*, respectively. The mixture was incubated at 30.0°. At various time intervals, 0.2 ml. aliquots were withdrawn and pippetted into 0.0265 *M* acetate buffer at pH 5.1 of a specified volume*, 1 ml. of this solution being measured by colorimetric ninhydrin method as described by Rosen (*10*)**. Calculation of the data was essentially similar to that of Schwartz and Engel (*13*). The color yields of amino acids and peptides based on L-leucine as 100 per cent are shown in Table I.

Synthesis of Peptide Derivatives

Gly_n (n=2, 3, 4 and 5)—These compounds were prepared as described in the literature (*14*).

* 1 ml. of this solution in the case of Gly_n-L-Tyr did not show any change in its ninhydrin color producibility even after leaving for 24 hours in a refrigerator which means that the enzyme was actually inactivated.

** The color of all glycyl peptides listed in Table I with ninhydrin gave maximal values after the first 5 minutes at 100° (*11*, *12*).

TABLE I

Per Cent Color Yield of the Compounds Based on L-*Leucine* = 100 *per cent*

Experimental details are described in the literature (*10*).

Substance	Color yield (*per cent*)	Substance	Color yield (*per cent*)
Gly-L-Tyr	93	Gly	100
Gly_2-L-Tyr	86	Gly_2	89
Gly_3-L-Tyr	84	Gly_3	82
Gly_4-L-Tyr	86	Cly_4	84
Gly_5-L-Tyr	85	Gly_5	79
L-Tyr	98		

CbzGly-L-*Tyr*—Carbobenzoxyglycyl-L-tyrosine ethyl ester obtained by the mixed anhydride method was saponified as described in the literature (*15*). m.p. 107° (m.p. 107° (*15*)).

CbzGly$_n$ (*n*=*3, 4 and 5*)—The procedure of Bergmann *et al.* (*16*) for the preparation of $CbzGly_3$ and $CbzGly_4$ was simplified by the use of the mixed anhydride method. $CbzGly_3$ was thus obtained from CbzGly and GlyGly in a yield of 64 per cent; m.p. 195° (m.p. 196° (*16*)). $CbzGly_4$ was made from $CbzGly_2$ (*17*) and GlyGly in a yield of 65 per cent; m.p. 228° (m.p. 230° (*16*)).

After the mixed anhydride of $CbzGly_2$ was coupled with a solution of GlyGlyGly in 1 *N* NaOH, the mixture was acidified with 2 *N* HCl. The resulting $CbzGly_5$ was recrystallized from aqueous methanol; yield, 53 per cent; m.p. 254–256°.

$C_{18}H_{23}O_8N_5$ (437.4): Calcd. N 16.0
Found N 15.9

As $CbzGly_5$ did not dissolve even in dimethylformamide, the mixed anhydride of the compound could not be prepared.

CbzGly-L-*TyrOBz*—The mixed anhydride from CbzGly (0.01 mole), isobutyl chlorocarbonate (0.01 mole), triethylamine (0.01 mole), and toluene (20 ml.) was prepared, and coupled with a mixture of L-TyrOBz *p*-toluenesulfonate (0.01 mole) (*18*), triethylamine (0.01 mole), and chloroform (20 ml.). The mixture was left overnight, and the crystals appeared after the washings with water, 4 per cent bicarbonate solution, 2 per cent HCl, and water by means of decantation. It was recrystallized from ethyl acetate-ether-petroleum ether. Yield, 73 per cent; m.p. 101°; $[\alpha]_D^{14}$ +3.5° (c 2, in DMF).

$C_{26}H_{26}O_6N_2$ (462.5): Calcd. C 67.5, H 5.7, N 6.1
Found C 67.7, H 5.7, N 6.2

Gly-L-*Tyr*—The above compound (0.004 mole) suspended in a mixture of methanol (30 ml.), acetic acid (8 ml.), and water (4 ml.) was treated with hydrogen in the presence of palladium black. The filtrate from the catalyst was evaporated *in vacuo* repeatedly, water being added. The remaining crystals were collected and recrystallized from water-ethanol. When a sample was dried *in vacuo* over P_2O_5 at 110°, it lost 23.5 per cent in weight, calculated for 4 moles of water, 23.2 per cent. Yield, 77 per cent; m.p. 174–176° (decomp.) (m.p. 165° (*19*)); $[\alpha]_D^{14}$ +44.7° (c 2, in water) ($[\alpha]_D^{25}$ +43.7° (*20*)).

$C_{11}H_{14}O_4N_2$ (238.2): Calcd. C 55.5, H 5.9, N 11.8
Found C 55.5, H 5.7, N 11.5

CbzGly$_2$-L-*TyrOBz*—The mixed anhydride of $CbzGly_2$ (0.01 mole) in tetrahydrofuran

(20 ml.) was coupled with L-TyrOBz in the same manner as that for CbzGly-L-TyrOBz. The crude crystals were recrystallized from acetone-ether. Yield, 69 per cent; m.p. 161°; $[\alpha]_D^{14}$+2.4° (c 2, in DMF).

$C_{28}H_{29}O_7N_3$ (519.5): Calcd. C 64.7, H 5.6, N 8.1
Found C 64.9, H 5.5, N 8.3

Gly_2-L-Tyr—The compound was prepared from the above ester as described in the case of Gly-L-Tyr. It was recrystallized from water-ethanol. Yield, 84 per cent; m.p. 232–234° (decomp.) (m.p. 218–220° (*21*)); $[\alpha]_D^{14}$+43.2° (c 2, in water) ($[\alpha]_D^{20}$+42.3° (*21*)).

$C_{13}H_{17}O_5N_3$ (295.3): Calcd. C 52.9, H 5.8, N 14.2
Found C 52.9, H 6.0, N 14.3

CbzGly$_3$-L-TyrOBz—Dimethylformamide (20 ml.) was used instead of toluene or tetrahydrofuran for the preparation of the mixed anhydride of $CbzGly_3$. The crude product obtained was recrystallized from acetone-ether. Yield, 71 per cent; m.p. 179°; $[\alpha]_D^{14}$+2.7° (c 2, in DMF).

$C_{30}H_{32}O_6N_4$ (576.6): Calcd. C 62.5, H 5.6, N 9.7
Found C 62.2, H 5.6, N 9.8

Gly_3-L-Tyr—The compound was prepared from the above ester in the usual manner and crystallized from water-ethanol with 3 moles of water. When it was dried at 110°, it lost 9.1 per cent weight, calculated for 2 moles of water for Gly_3-L-Tyr trihydrate, 8.9 per cent. Yield, 76 per cent; m.p. 180–184° (decomp.); $[\alpha]_D^{14}$+39.7° (c 2, in water).

$C_{15}H_{20}O_6N_4 \cdot H_2O$ (370.4): Calcd. C 48.6, H 6.0, N 15.1
Found C 48.5, H 5.9, N 14.9

CbzGly$_4$-L-TyrOBz—The compound was prepared from $CbzGly_4$ in the same manner as that for $CbzGly_3$-L-TyrOBz. The crude crystals were recrystallized from a large amount of hot methanol. Yield, 73 per cent; m.p. 149°; $[\alpha]_D^{14}$+0.7°(c 2, in DMF).

$C_{32}H_{35}O_9N_5$ (633.6): Calcd. C 60.7, H 5.6, N 11.1
Found C 60.7, H 5.7, N 10.9

Gly_4-L-Tyr—The compound was prepared from the above ester and crystallized from water-ethanol with 2 moles of water. When it was dried at 110°, it lost 4.4 per cent, calculated for 1 mole of water for Gly_4-L-Tyr dihydrate, 4.1 per cent. Yield, 67 per cent; m.p. 199–202°; $[\alpha]_D^{14}$+36.6° (c 2, in water).

$C_{17}H_{23}O_7N_5 \cdot H_2O$ (427.4): Calcd. C 47.8, H 5.9, N 16.4
Found C 47.6, H 5.7, N 16.4

TritylGly-L-TyrOBz—The mixed anhydride from tritylGly (0.05 mole) (*22*), isobutyl chlorocarbonate (0.05 mole), triethylamine (0.055 mole), and chloroform (100 ml.) was coupled with L-TyrOBz (0.05 mole) in chloroform as described in the case of CbzGly-L-TyrOBz. The reaction mixture was washed with water, 0.2 *N* acetic acid, 0.2 *N* bicarbonate solution, and water; the organic layer was dried over Na_2SO_4, and concentrated *in vacuo*. The crude crystals obtained were recrystallized from ethanol-petroleum ether. Yield, 67 per cent; m.p. 184°; $[\alpha]_D^{14}$−14.5° (c 2, in DMF).

$C_{37}H_{34}O_4N_2$ (570.7): Calcd. N 4.9
Found N 4.9

Gly-L-TyrOBz hydrochloride—A solution of the above ester (0.02 mole) in 0.5 *N* HCl in methanol (42 ml.) was heated for 2 minutes in a water bath. Evaporation *in vacuo* and trituration of the residue with ether resulted in crystallization of the desired compound. It was recrystallized from ethanol-ether. Yield, 96 per cent; m.p. 202–203°; $[\alpha]_D^{14}$+1.3°

(c 2, in water).

$C_{18}H_{20}O_4N_2.HCl$ (364.8): Calcd. N 7.1
Found N 7.0

CbzGly$_5$-L-TyrOBz—This compound was prepared from CbzGly$_4$ and Gly-L-TyrOBz in the usual manner. The crude crystals were recrystallized from a large amount of hot methanol or DMF-ether-petroleum ether. Yield, 76 per cent; m.p. 194–195°; $[\alpha]_D^{14}+1.1°$ (c 2, in DMF).

$C_{34}H_{38}O_{10}N_6$ (690.7): Calcd. C 59.1, H 5.6, N 12.2
Found C 58.8, H 5.6, N 12.3

Gly$_5$-L-Tyr—This compound was prepared from the above compound and crystallized from water-ethanol with 3 moles of water. When it was dried at 110°, it lost 7.1 per cent in weight, calculated for 2 moles of water for Gly$_5$-L-Tyr trihydrate, 6.9 per cent. Yield, 78 per cent; m.p. 208–211° (decomp); $[\alpha]_D^{14}+25.7°$ (c 2, in water).

$C_{19}H_{26}O_8N_6\cdot H_2O$ (484.5): Calcd. C 47.1, H 5.8, N 17.4
Found C 47.3, H 5.9, N 17.7

RESULTS AND DISCUSSION

pH Optimum for Hydrolysis by Carboxypeptidase

For the comparison of hydrolytic susceptibility of the peptides Gly$_n$-L-Tyr (n=1∼5), it seems desirable to determine the rates of hydrolysis of the substrates at their optimum pH. Comparative measurements of the effect

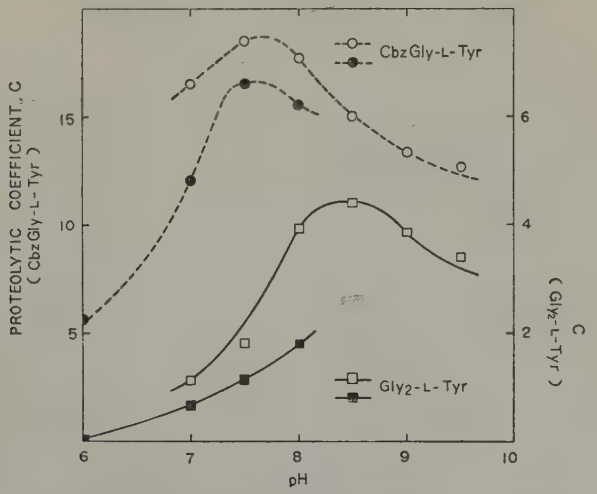

FIG. 1. The pH dependence of the hydrolysis of CbzGly-L-Tyr and Gly$_2$-L-Tyr by CPase in 0.1 *M* LiCl at 30°. The substrate concentration was 0.01 *M*. ●, ■; 0.05 *M* phosphate buffer. ○, □; 0.05 *M* with respect to the amine component of Tris—HCl buffer.

of pH on the peptidase activity of CPase were made with Gly$_2$-L-Tyr as a representative substrate, and the results were shown in Fig. 1, an optimum

pH of the reaction appearing to near 8.5.

As seen from Fig. 1, the values of the proteolytic coefficient in phosphate buffer were lower than that in Tris buffer within the range measured (pH 7.0~8.0). This results may be attributed to the inhibitory action of phosphate, since Smith and Hanson (*23*) have noted that CPase is inhibited by phosphate, and other inorganic salts which combine with metal, which is an integral part of the enzyme molecule (*24*).

For the comparison of hydrolytic rates of the peptides Gly_n-L-Tyr (n=1~5), a Tris buffer at pH 8.5 was used throughout the experiments.

For comparison, data for CbzGly-L-Tyr are included in Fig. 1. An optimum pH of the reaction appears to be near 7.6, while Smith and Hanson (*23*) reported that an optimum pH of CbzGly-L-Leu falls in the pH range 7.4~7.8.

It is of interest that the pH optimum of Gly_2-L-Tyr is significantly higher than that of CbzGly-L-Tyr. The similar facts were reported in regard to chymotryptic activity towards glycyl-L-tyrosinamide and benzoyl-L-tyrosinamide (*25*, *26*), and action of CPase towards D-Leu-L-Tyr and acyl dipetide (*5*). Such results would be expected if the observed proteolytic coefficients were dependent not only on the effect of pH on the enzyme but also on the concentration of available substrate at a given pH as Yanari and Mitz had suggested previously (*5*). If only the species of peptides and peptide amides with an uncharged amino group were the true substrate of CPase and chymotrypsin, respectively, the hydrolysis at a higher pH would tend to be high in relation to that at a lower pH.

Proteolytic Coefficients in Various Initial Substrate Concentrations

In order to compare the hydrolytic rates of the peptides Gly_n-L-Tyr (n=1~5) by CPase, the values of proteolytic coefficients in various substrate concentrations and C_{max} were determined (*1*, *25*).

The compounds were tested at 0.025 *M*, and 0.015 *M*, 0.01 *M* and 0.005 *M* substrate concentrations. Representative data for the hydrolysis of three substrates at 0.01 *M* substrate concentration are shown in Table II as an example. Under the conditions given in the section of *methods*, it was found that the hydrolysis of the substrates tested followed approximately first order kinetics, however the proteolytic coefficients had the tendency to decrease very slightly with increasing the time and per cent of hydrolysis in all cases except Gly-L-Tyr. The hydrolysis of Gly-L-Tyr followed the kinetics of first order within the extent of experimental error as shown in Table II. Since plots of apparent proteolytic coefficient of the substrates *versus* time was almost linear as may be seen in Table II, the proteolytic coefficients at the zero time calculated graphically, which are shown in Table III, were taken for the comparison of sensibility of the substrates.

A summary of all coefficients determined are given in Table III, and just as in the case of aminoacyl-L-tyrosinamides with chymotrypsin (*25*), the coefficients vary with the concentrations of the substrates. As seen from

Table II

Example in Measurements of Hydrolysis of CbzGly-L-Tyr, Gly-L-Tyr and Gly_2-L-Tyr by Carboxypeptidase

Substrate concentration, 0.01 *M*; pH 8.5 (0.05 *M* Tris buffer); 0.1 *M* LiCl; temperature, 30°.

Substrate	Enzyme concentration (*mg. protein N per ml.*)	Time (*min.*)	Hydrolysis (*per cent*)	Proteolytic coefficient
CbzGly-L-Tyr[1]	0·000207	9.5	7.9	18.2
		20.0	15.9	18.2
		35.0	25.7	17 8
		51.4	35.0	17.6
Gly-L-Tyr[2]	0.187	53	12.3	0.00575
		140	29.7	0·00585
		258	46.9	0.00570
		365	59.8	0.00580
Gly_2-L-Tyr	0.00235	10.5	21.1	4.17
		20.0	36.0	4.12
		33.0	52.0	4.11
		42.8	60.2	3.97

1) Reactions were carried out at pH 7.5 (0.05 *M* Tris buffer).
2) 0.2 *M* LiCl was used.

Table III

Proteolytic Coefficients of CbzGly-L-Tyr and Gly_n-L-Tyr ($n=1\sim5$) in Various Initial Substrate Concentrations

pH 8.5 (0.05 *M* Tris buffer); 0.1 *M* LiCl; enzyme concentration, 0.00235 mg. N/ml.; temperature, 30°.

Substrate	Proteolytic coefficient			
	0.025 *M*	0.015 *M*	0.01 *M*	0.005 *M*
CbzGly-L-Tyr[1]	8.4	13.3	18.3	27.2
Gly-L-Tyr[2]	0.0023	0.0029	0.0058	0.011
Gly_2-L-Tyr	2.0	3.0	4.2	6.2
Gly_3-L-Tyr	2.0	3.0	4.2	6.2
Gly_4-L-Tyr	2.1	3.2	4.5	6.4
Gly_5-L-Tyr	1.7	3.2	4.1	5.8

1) Reactions were carried out at pH 7.5 (0.05 *M* Tris buffer). Enzyme concentration was 0.000207 mg. N per ml.
2) 0.2 *M* LiCl was used. Enzyme concentration was 0.187 mg. N per ml.

Table III, the values of coefficients of the substrates Gly_n-L-Tyr ($n = 2\sim5$) at a given substrate concentration were approximately similar to each other. In this point, a further discussion will be noted in the next section.

Reaction Kinetics and C_{max}

Measurements of initial rates of hydrolysis at different concentrations of Gly_n-L-Tyr (n=1~5), and the use of the plotting method of Lineweaver

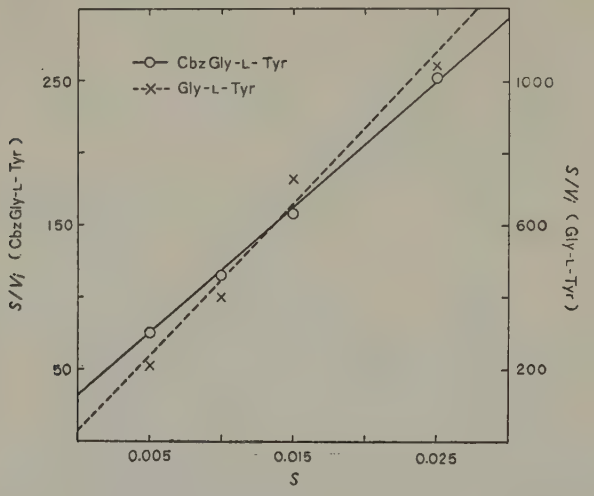

FIG. 2. A plot of initial substrate concentration S divided by initial velocity V_i *versus* S for the hydrolysis of CbzGly-L-Tyr (pH 7.5, 0.000207 mg. N per ml.) and Gly-L-Tyr (pH 8.5, 0.187 mg. N per ml.) at 30°.

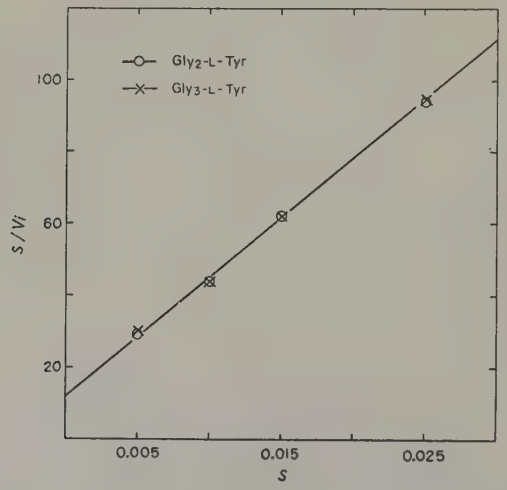

FIG. 3. A plot of initial substrate concentration S divided by initial velocity V_i *versus* S for the hydrolysis of Gly_2-L-Tyr and Gly_3-L-Tyr at 30°, pH 8.5, and 0.00235 mg. N per ml.

and Burk, gave the values of $K_m=(k_2+k_3)/k_1$ at pH 8.5 and 30° for the hydrolysis of the substrates by CPase. Plots for the hydrolysis of Gly_n-L-Tyr

(n=1∼3) are shown in Fig. 2 and 3 as examples. The magnitudes of k_3 defined as the rate constant for the conversion of enzyme-substrate complex to enzyme and products were determined by plots of Lineweaver and Burk or by means of the integrated form of the Michaelis-Menten equation (*17*, *20*). The apparent maximum proteolytic coefficients will be calculated by the equation of $C_{max}=k_3/2.3K_m$ (*20*).

The values of K_m, k_3 and C_{max} are shown in Table IV.

Table IV

Kinetic Constants of CbzGly-L-Tyr and Gly_n-L-Tyr (n=1∼5)

pH 8.5 (0.05 *M* Tris buffer); 0.1 *M* LiCl; temperature, 30°.

Substrate	K_m (10^{-3} *M*)	k_3[3]	C_{max}
CbzGly-L-Tyr[1]	3.8	0.56	64
Gly-L-Tyr[2]	0.68	0.00013	0.083
Gly_2-L-Tyr	3.4	0.129	16.5
Gly_3-L-Tyr	3.3	0.127	16.7
Gly_4-L-Tyr	3.7	0.137	16.1
Gly_5-L-Tyr	3.5	0.136	16.9

1) Reactions were carried out at pH 7.5 (0.05 *M* Tris buffer).
2) 0.2 *M* LiCl was used.
3) In *M*/liter/min./mg.N/ml.

As shown in Table III and IV, the introduction of Gly_n (n=1∼4) to N-terminal position of Gly-L-Tyr causes a marked increase in the hydrolytic rate; Gly_n-L-Tyr (n=2∼5) were hydrolyzed approximately 200 times rapidly than Gly-L-Tyr. It was observed that there is little difference among the values of K_m, k_3 and C_{max} of Gly_n-L-Tyr (n=2∼5), the similar mechanism of enzymatic action of CPase on the peptides Gly_n-L-Tyr (n=2∼5) being suggested. In other words, the changes in the environmental conditions imposed on the sensitive bond by di-, tri-, tetra- and penta-glycyl residues have little effect on the rates of release of L-tyrosine from the peptides polyglycyl-L-tyrosine.

Yanari and Mitz pointed that Gly-L-Tyr, the very poor substrate for hydrolysis by CPase, may interact with the active site of the enzyme, and form the extremely stable complex (*5*). The lower values of K_m and k_3 of Gly-L-Tyr than that of other substrates in Table IV support their suggestion.

Paperchromatography of Reaction Mixture

In addition to measurements by the colorimetric ninhydrin method of the amount of amino acids and peptides, the reaction was also followed by transferring 5∼20 μl. samples on filter paper. In all cases, only the simple hydrolysis of the substrates occurred, yielding L-tyrosine and corresponding polyglycine Gly_n (n=1∼5). Even in the after stage of the reaction (up to 10

hours), it was shown that Gly_n (n=2∼5) were not hydrolyzed by the enzyme.

The R_f values of the reference compounds in two different solvent systems are given in Table V.

TABLE V

R_f Values of Reference Compounds

The compounds were chromatographed on Toyo Roshi No. 50 paper using *n*-butanol : acetic acid : pyridine : water (15 : 3 : 10 : 12, by vol.) and *n*-butanol : acetic acid : water (4 : 1 : 2, by vol.). The ascending technique was applied.

Substance	R_f	
	(15 : 3 : 10 : 12, by vol.)	(4 : 1 : 2, by vol.)
L-Tyr	0.51	0.35
Gly-L-Tyr	0.50	0.37
Gly	0.25	0.13
Gly_2-L-Tyr	0.47	0.31
Gly_2	0.22	0.13
Gly_3-L-Tyr	0.43	0.25
Gly_3	0.22	0.12
Gly_4-L-Tyr	0.41	0.21
Gly_4	0.21	0.09
Gly_5-L-Tyr	0.37	0.15
Gly_5	0.20	0.07

SUMMARY

1. A number of the peptides Gly_n-L-Tyr in which n are 1, 2, 3, 4 and 5 have been synthesized and tested as substrates for carboxypeptidase.

2. By the use of Gly_2-L-Tyr, the pH optimum of hydrolysis was found to be near 8.5. For comparison, the pH optimum for CbzGly-L-Tyr was checked and found to be near 7.6.

3. The values of proteolytic coefficients at the initial substrate concentrations of 0.025 *M*, 0.015 *M*, 0.01 *M* and 0.005 *M*, and C_{max}, determined at 30° and in Tris buffer at pH 8.5, were taken as measures of the relative susceptibility of hydrolysis of the substrates by carboxypeptidase. Chromatographic analysis of the reaction mixture proved the simple hydrolysis of the substrates to L-tyrosine and corresponding polyglycine Gly_n (n=1∼5).

4. The same hydrolytic rates by carboxypeptidase of the substrates Gly_n-L-Tyr (n=2∼5) were observed; the C_{max} of the substrates were 16∼17. Gly-L-Tyr was hydrolyzed approximately 1/200 times slowly than Gly_n-L-Tyr (n=2∼5).

The authors wish to thank Prof. S. Shibuya for his interest, and Dr. A. Tanaka for his discussion in this study.

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THE INFLUENCES OF INSULIN HYPOGLYCAEMIC COMA, REPEATED ELECTROSHOCKS, AND CHLORPROMAZINE OR β-PHENYLISOPROPYLMETHYLAMINE ADMINISTRATION ON THE FREE AMINO ACIDS IN THE BRAIN

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Many papers have been published from the standpoint that the etiology of mental diseases would be clarified by investigating the influences of the therapies effective to mental diseases and of drugs producing mental disorders on the metabolism of brain.

On the amino acids metabolism, however, the change of the glutamic acid group has been chiefly discussed, and little study has been done on the effects of such treatments upon the whole pattern of amino acids in the brain. Several experiments (*1*, *2*) have indicated that the total amino acid content of the brain tends to be maintained at a steady level, though large changes take place in the individual amino acids. From these facts, it would appear that the change of one component of the free amino acid pool of the brain must be accompanied with the complementary changes of the others. When we take up the subjects of amino acid metabolism in the brain, therefore, it would be essential that the pattern of amino acids as a whole should be investigated. Moore and Stein (*3*) have reported that the ion-exchange chromatography gives the most satisfactory results for such analysis of the spectra of amino acids.

The present study has been undertaken to obtain the changes of the free amino acid pattern in the brain of rats due to insulin hypoglycaemic coma, electroshock, and chlorpromazine or β-phenylisopropylmethylamine administration by application of the ion-exchange chromatographic procedure.

EXPERIMENTALS

Materials—In order to eliminate individual difference in the animals, white male rats, weighing about 100 g. and fed on wheat and vegetables, were employed in the following experiments.

Normal Rats, as the control, fed until just before the examination without any treatment.

Fasting, abstained from feed for 24 hours before the decapitation.

Insulin Hypoglycaemic Coma—The fasting rats were injected subcutaneously in a dose of

100 units per kg. body weight of insulin. Two hours after, they were killed in the comatose state.

Repeated Electroshocks—Being loaded with electric stimulations of 25 volt-3 seconds, three times daily, for about 10 days, rats were decapitated at 24 hours after the last stimulation in so irritable state that they were ready to bite hearing just a faint sound.

Chlorpromazine were daily administered in a dose of 10 mg. per kg. body weight by intraperitoneal injection for 30–60 days.

β-Phenylisopropylmethylamine (Methylpropamine J. P.), were injected in a dose of 6 mg. per kg. daily for 30–60 days.

The administration of these two drugs produces no marked changes in rat behavior, though the group administered with the former increased less in body weight than the latter. These two groups were come into use respectively at 24 hours and 48 hours after the last injection.

Preparation of Brain Extracts—The animals were sacrificed by decapitation without any anaestheses, and brains were isolated, immediately frozen in dry-ice aceton, and only the cerebra to be analyzed were weighed by torsion balance. The samples were ground in Potter-Elvehjem glass homogenizers with a 30-fold amount of 90 per cent ethanol, and the ethanol precipitate was removed by centrifugation. The ethanol extract was evaporated to dryness over water bath at 50°. The dried residue was dissolved with 0.2 *N* sodium citrate buffer at pH 2.2, and this samples were again frozen until analyses.

Chromatographic Analysis—The samples were analyzed on columns of Dowex 50-×4 150×0.9 cm., by Moore and Stein's procedure (*3*). Every 2 ml. of effluent fraction was collected. The concentrations of the amino acids in the effluent fractions were determined photometrically by the modified ninhydrin reagent (*4*) for 1 ml. of each fraction. The combination of the remaining solutions, corresponding to the ninhydrin positive substance in the peak, was identified by the method of paperchromatography (*5*). On *N*-acetylaspartic acid, remaining 1 ml. of such fractions were determined by modified ninhydrin method after acid hydrolysis. In the chromatographic procedures, taurine and phosphoethanolamine were overlapped. So the eluate containing these two substances was chromatographed on paper with 90 per cent methyl-cellosolve as the solvent system.

Total Amino Acid Nitrogen Contents of the samples were obtained by the modified ninhydrin method.

RESULTS

The pattern of free amino acids and related compounds in the brain of male rats in normal, fasting, insulin hypoglycaemic coma, repeated electroshocks and administration of chlorpromazine or β-phenylisopropylmethylamine is shown in Table I. Values for the amino acids contained in a small concentration, were determined from the combined samples from 3–5 rats, while the other compounds could usually be analyzed by using about 1 g. of cerebrum from a rat.

In fasting, there is no marked difference from the normal pattern, but glycerophosphoethanolamine content is slightly decreased and aspartic acid and threonine tend to increase.

It is obvious that insulin hypoglycaemic coma produces the significant increase of aspartic acid content and the lower concentrations of glutamic acid and alanine. Both the fall of glycerophosphoethanolamine and the small rise of threonine due to insulin treatment, however, were also found

in fasting.

In repeated electroshocks, no marked changes in concentrations of almost all compounds take place, except for the slight increase of glutamic acid content.

TABLE I

The Concentrations of Ninhydrin Positive Constituents in the Brain of the Rat under Normal and Various Conditions

Values expressed as mg. per 100 g. wet weight.

Compound	Normal (5)*	Fasting (1)	Insulin hypoglycaemic coma (2)	Repeated electro shocks (2)	Chlorpromazine administered (3)	β-Phenylisopropylmethylamine administered (3)
Amino acid N	43.5	43.5	44.7	46.8	—	—
Glycerophosphoethanolamine	17.8	10.5	11.8	18.7	11.8	13.1
Phosphoethanolamine	19.8	26.0	23.2	21.9	19.5	24.0
Taurine	49.0	61.9	59.9	58.2	71.3	60.1
N-Acetylaspartic acid	75.1	84.1	71.6	74.3	7 .0	101.9
Urea**	50	40	50	60	40	40
X_2***	0.011	0.014	0.016	0.021	0.021	0.021
Aspartic acid	30.0	34.9	49.4	31.7	35.4	33.0
Threonine	3.6	6.5	6.6	5.6	6.1	5.4
Serine	10.1	11.2	9.8	11.9	11.5	9.5
Glutamine**	70	120	90	130	130	130
Glutamic acid	147.0	152.8	128.1	161.8	179.2	151.9
Glycine	6.8	7.8	7.4	6.1	6.8	6.2
Alanine	5.0	4.1	1.9	4.6	5.1	4.3
Glutathione**	25.9	—	—	—	27.8	18.9
Isoleucine	1.6	—	—	—	—	—
Leucine	1.7	—	—	—	—	—
γ-Aminobutyric acid	22.1	—	—	—	24.0	21.4
Lysine	1.8	—	—	—	—	—
Histidine	1.4	—	—	—	—	—
Arginine	1.9	—	—	—	—	—

* Parenthesized passage represents the number of animals.

** Approximate values (see text).

*** Leucine equivalents-mM concentration.

— Not examined.

Administration of β-phenylisopropylmethylamine also causes only the increase of *N*-acetylaspartic acid concentration. Chlorpromazine, however, produces the increase of glutamic acid, aspartic acid, γ-aminobutyric acid,

and taurine content, besides the decrease of glycerophosphoethanolamine.

Although the total amino acid nitrogen contents have not been determined in every case, its level was relatively stable during insulin coma and in electroshocks.

The recoveries of glutamine, glutathione and urea are unsatisfactory (*3*, *6*), so the data of them shown in the table are quantitatively not reliable.

The previously reported unknown substance, X_2, which is an interesting substance in comparative biochemistry (*6*), is shown in leucine epuivalents-mM concentration. The relative concentration of this substance shows no marked change in these treatments.

DISCUSSION

The values found in the brain of normal male rats are in agreement with those determined by other authors (*7–10*) with exception of a few substances. The values of threonine and lysine which Schurr *et al.* (*7*) determined by microbiological method, reached many times over ours, that, however, were in the same level as others observed with ion-exchange chromatograms (*11*).

The effects of age, sex, diet, exercise and temperature on the free amino acids in the brain have been previously studied (*12–15*). In the present work, however, the changes due to the above factors can be excluded out of the subject. Although Schurr *et al.* (*16*) reported the rise of threonine in fasting, its increase obtained in the present study was not large enough to confirm his results.

During insulin hypoglycaemic coma, aspartic acid content markedly increased (+0.145 mM per 100 g.), and glutamic acid (−0.127 mM per 100 g.) and alanine (−0.035 mM per 100 g.) decreased, compared with the pattern in fasting. Cravioto *et al.* (*1*) have also reported that insulin coma increases aspartic acid content and reduces γ-aminobutyric acid and glutamine content. Dawson (*17*) suggested that the fall of total amino acid nitrogen level is caused by the decrease of glutamic acid content, but Cravioto's and our researches have indicated that insulin coma produces no marked change in the total amino acid nitrogen level. Moreover, the decrease of glutamic acid was approximately equivalent to the increase of aspartic acid in molar concentration. Cravioto *et al.*, therefore, suggested that the transamination reaction would play a significant role in this case. Although such a direct amino group transfer may lead to this phenomenon, there may be associated with this change the indirect pathway at the same time, through which glutamic acid dehydrogenase converts glutamic acid to α-ketoglutaric acid to be mobilized for carbohydrate metabolism in hypoglycaemia and liberated ammonia is reaminated to oxalacetic acid. As to the decrease of alanine content, too, various dynamic pathways may be suggested. At this time, however, there is no conclusive evidence that insulin activates such a metabolic pathway both *in vivo* and *in vitro*.

It is interesting that both the repeated electroshocks and the administration of chlorpromazine, admitted to be effective to mental disorders, tend increase glutamic acid content.

β-Phenylisopropylmethylamine, whose chronic addition produced schizophrenia-like psychosis in Japan, increased only *N*-acetylaspartic acid content without any change of the other constituents. *N*-acetylaspartic acid is an important substances that serves to make up part of the anion deficit known to exist in nervous tissue, but quantitative accuracy for this substance by our estimation method is not always satisfactory, therefore the change is to be further studied.

That insulin coma and chlorpromazine greatly lower the phospholipid turnover in the brain has been previously found (*18–21*). Considering this facts, it is interesting that the glycerophosphoethanolamine content is decreased by insulin coma and chlorpromazine administration.

As described above, significant change of the free amino acid pattern in the brain was found only in insulin hypoglycaemic coma, and the other prolonged administrations caused no marked changes. But early studies (*22*, *23*) have indicated that convulsion, chlorpromazine, β-phenylisopropylmethylamine produce the respective changes of the pattern, so far as they are abruptly administered. Such facts may probably be due to the biological homeostatic mechanism.

SUMMARY

1. The present study has been undertaken to determine, as the first step, the pattern of free amino acids and related compounds in the brain of the normal rat. Secondarily, the changes of the pattern due to fasting, insulin hypoglycaemic coma, repeated electroshocks, and administration of chlorpromazine or β-phenylisopropylmethylamine have been studied by chromatography on Dowex 50-×4 columns.

2. During insulin hypoglycaemic coma, free aspartic acid concentration considerably increased, while free glutamic acid and alanine concentrations decreased. Both the fall of glycerophosphoethanolamine and the rise of threonine taking place in this treatment were also found in fasting rat.

3. In the irritable stage produced by repeated electroshocks, the glutamic acid content slightly increased without any change in other constituents.

4. Repeated injection of chlorpromazine for 30–60 days increased glutamic acid, aspartic acid, γ-aminobutyric acid and taurine content, besides a decrease of glycerophosphoethanolamine.

5. Repeated injection of β-phenylisopropylmethylamine produced no marked changes in the pattern, but only the increase of *N*-acetylaspartic acid concentration was observed.

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THE METABOLISM OF ACETATE IN CHLORELLA CELLS

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As a preliminary research for this series of studies, the present author investigated the capacity of *Chlorella ellipsoidea* for oxidizing various organic substances, including carbohydrates and organic acids*. Among the substances studied, glucose and acetate were found to be most readily metabolized by the alga. The purpose of the present study was to follow the metabolism of these substrates in more detail. The process of oxidative assimilation of these substances was also followed. Special stress was laid on the investigation of acetate metabolism, since it was discovered in the course of the present study that this particular acid was assimilated with most rapidity and with strikingly high efficiency by the algal cells.

The effects of poisons such as cyanide and 2,4-dinitrophenol on the respiration, on the one hand, and on the assimilatory process, on the other hand, were also investigated. The rather unexpected discovery, in this connection, that carbon dioxide also exerts an inhibitory effect on the metabolism of the algal cells will be reported.

The changes in the metabolic activities during the cellular life-cycle were also investgated. The results obtained are discussed with reference to the general pattern of metabolism of *Chlorella* cells.

METHODS

The test materials used in this study were "dark cells" of *Chlorella ellipsoidea*, obtained by the method of synchronous culture developed in the Tokugawa Biological Institute by Tamiya and coworkers (*1*). To describe this briefly, the alga was cultured at 25° for 7 days in a medium which had the following composition: 0.05 *M* KNO_3, 0.01 *M* $MgSO_4 \cdot 7H_2O$, 0.01 *M* KH_2PO_4, 10^{-5} *M* $FeSO_4 \cdot 7H_2O$ (pH 5.3–5.4) per liter of tap water. CO_2-enriched air was constantly bubbled through the culture. For the first five days, the culturing was carried out under intense illumination of about 5000–15000 lux. The culture was then transferred to the dark in order to obtain a uniformly synchronized yield of dark cells. The cells were harvested and washed three times with distilled water on a centrifuge and suspended in a phosphate buffer of pH 7.0 (*M*/25). Gas exchange was measured by the usual manometric technique of Warburg. Most of the manometric measurements were made in a respiration flask with KOH in the center well and 0.5 ml. of substrate in the side arm. The final concentrations of acetate and glucose were 10^{-2} *M* unless otherwise stated.

* to be published elsewhere.

Isotope experiments were carried out in the dark in a glass vessel provided with a glass stopper (Fig. 1). Radioactive acetates labelled at the carboxyl or methyl position were used at an isotopic concentration of 0.45 μc per vessel (2.5–5.0 ml. reaction mixture). The cells were incubated with the substrate for 2–4 hours, and the reaction was stopped by the addition of concentrated acetic acid (see Fig. 1). The cells were immediately killed by this treatment. Carbon dioxide absorbed in KOH in the center well was precipitated as barium carbonate. To measure the amounts of incorporated C^{14}, the acid-treated cells were transferred to a dish of stainless steel, dried under an infra-red lamp, and the radioactivity was determined with the use of an end-window type Geiger-Müller tube. The radioactivity in the barium carbonate was measured by the similar procedure.

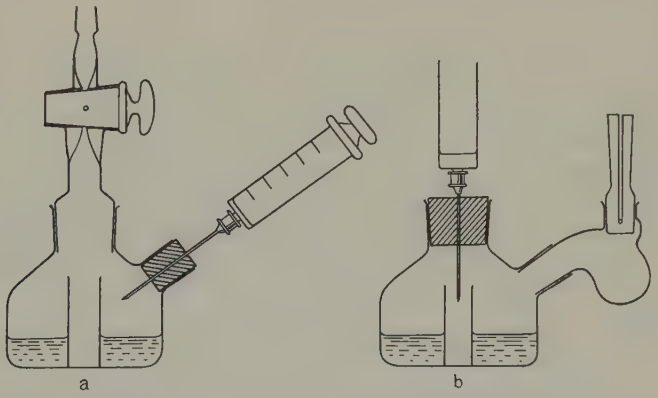

FIG. 1. Vessels used in experiments with radioactive carbon.

a) The reaction was started by injecting a given amount of labelled acetate through the rubber stopper. After given period of incubation, the reaction was stopped by injecting concentrated solution of (non-labelled) acetic acid through the same stopper.

b) In the experiments with non-labelled carbon dioxide in the gas-atmosphere, KOH solution was added to the center well at the end of the reaction period.

In CO_2-inhibition experiments, KOH was added in the center well, at the end of the indicated incubation period, and after the addition of concentrated acetic acid to the reaction mixture to remove from the solution all the dissolved carbon dioxide.

The cells incorporating C^{14} were removed at intervals and submitted to chemical fractionation into three parts by a modification of Schneider's method (*2*): 20 per cent acetic acid-soluble fraction; ethanol-ether (3 : 1)-soluble fraction; and the residue. Also the changes in the capacity for oxidative assimilation of the cells were followed along the course of the cellular life-cycle of *Chlorella*, according to the method of Tamiya *et al.*[7](*3*).

RESULTS

Fate of Carboxyl and Methyl Carbon of Acetate—The amounts of methyl and carboxyl carbon evolved as CO_2 and incorporated to the cells are separately presented in Tables I, and II. The results in Table I are reproduced in Fig. 2. The molarity was calculated from the specific activity of each sample

TABLE I

Fate of Methyl and Carboxyl Carbon of Acetic Acid

Carbon	Time (*min.*)	Total C $m\text{M} \times 10^{-7}$	CO_2 output		C incorporated*	
			$m\text{M} \times 10^{-7}$	%	$m\text{M} \times 10^{-7}$	%
Methyl carbon	10	449.9	9.0	2.0	440.9	98.0
	20	454.5	8.1	1.8	446.4	98.2
	30	618.9	15.8	2.6	603.0	97.4
	40	739.8	30.5	4.2	709.3	95.8
	90	2022.0	122.0	6.0	1900.0	94.0
Carboxyl carbon	10	456.4	26.0	6.0	416.8	94.0
	20	667.9	86.2	12.9	581.7	87.0
	30	866.0	146.0	16.8	720.7	83.2
	40	1011.3	226.9	22.4	784.4	77.6

Molarity of respective carbon is calculated from specific activity of the radioactive acetate used. Final concentration of acetate: 10^{-2} *M*.

* Total molarity of (labelled and non-labelled) carbon incorporated.

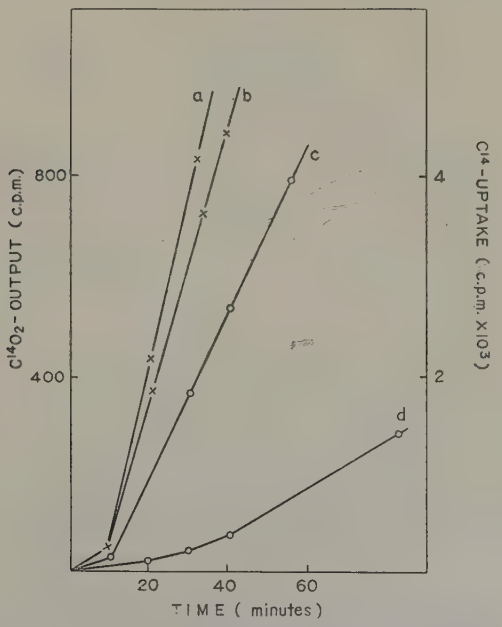

FIG. 2. Incorporation and CO_2-output with labelled acetate. a: incorporated C^{14} from methyl C^{14}, b: incorporated C^{14} from carboxyl C^{14}, c: $C^{14}O_2$ from carboxyl C^{14}, d: $C^{14}O_2$ from methyl C^{14}.

of labelled substrates. From Table I it will be seen that the carbon evolved as carbon dioxide originated almost exclusively from the carboxyl carbon of acetic acid. Indeed, it will be computed from the results in the table that 86 per cent of the CO_2-carbon evolved in a reaction period of 40 minutes, has arisen from carboxyl carbon of acetic acid. The carbon of incorporated material, on the other hand, is partly derived from the methyl and partly from the carboxyl group of the substrate, the corresponding figures for 40 minutes reaction period being about 1 : 1, as seen from Table I. Most prominent with the organism studied was the high degree of oxidative assimilation. Computation from the data given in Table II gives the following figures for the efficiency of the process involved;

Methyl-C assimilated : O_2 consumed = 0.81 : 1
Carboxyl-C assimilated : O_2 consumed = 0.57 : 1
Total-C assimilated : O_2 consumed = 1.38 : 1

The comparison of percentages of substrate carbon atoms discharged as CO_2, on the one hand, and assimilated on the other hand, as given in the two tables, also indicates a strikingly high grade of assimilation, especially with methyl carbon in earlier stages of incubation (see Table I).

The rates of C^{14} incorporation and C^{14} evolution from labelled acetic acid was found to be uninfluenced by illumination. This was true with respect to either the methyl or carboxyl carbon of the acid.

TABLE II

Balance Sheet of Oxidative Assimilation of Acetic Acid

Incubation time: 30 minutes.

		CO_2 output		C incorporated		Acetate consumed	O_2 uptake*
		$m\text{M} \times 10^{-7}$	%	$m\text{M} \times 10^{-7}$	%	$m\text{M} \times 10^{-7}$	$m\text{M} \times 10^{-7}$
Methyl carbon	A	3.15	3.2	95.3	96.8		118.0
	A′	(3.22)		(96.8)		(100)	(120)
Carboxyl carbon	B	48.1	31.1	106.0	69.0		118.0
	B′	(31.0)		(69.0)		(100)	„
	C	(34.2)	17.1	(165.8)	82.9	(200)	„

A and B: value obtained by isotopic technique, A′ and B′: values calculated by assuming acetate consumed as 100, C: Total of A′ and B′.

* O_2-uptake was calculated from the data in manometric experiments.

Distribution of Incorporated Carbon—The distribution of incorporated carbon atoms among the above-stated fractions of cell material was followed. Fraction I, the 20 per cent acetic acid-soluble fraction, consisted of organic acids and free amino acids, the largest portion, as high as 80 per cent of

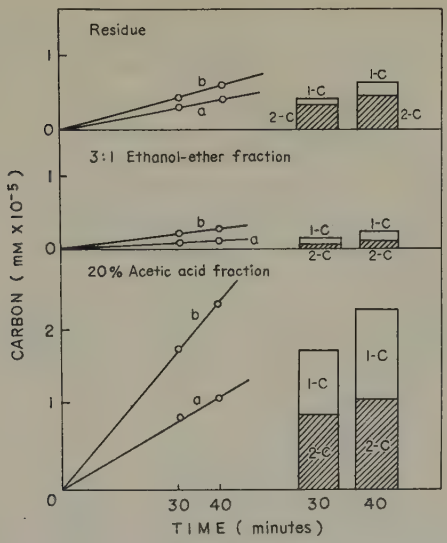

FIG. 3. Distribution of methyl and carboxyl carbon of acetate into various fractions of cell material.

a) incorporation of methyl C^{14} (2-C),

b) Total incorporation (1-C, 2-C).

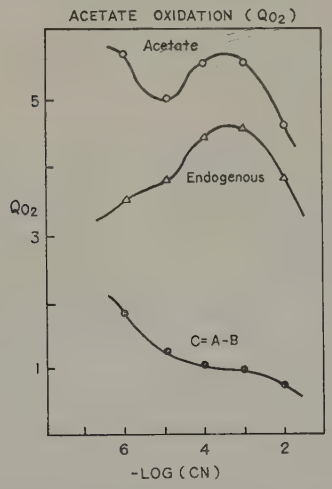

FIG. 4. Oxidation of acetate in the presence of cyanide. The dotted line at the left of the graph indicate the level of endogenous respiration at absence of cyanide.

the incorporated carbon, being recovered in this fraction. It was found that the carbon atoms of Fraction I were derived about equally from the methyl and carboxyl carbon of the substrate (Fig. 3). Fraction II, ethanol-ether (3:1)-soluble fraction, comprising lipids, pigments of the carotene

group and chlorophylls, and certain contaminants of organic acids was found to be relatively small (about 7 per cent). In this case also, the chance for methyl and carboxyl carbon of the substrate to enter the fraction seems to be equal. Fraction III, the residue, including the bulk of the proteins,

TABLE III

Rate of O_2-Uptake in the Presence of Inhibitor During Oxidation of Acetate

Inhibitor (*min.*)		Oxygen-uptake (*μl.*)					
		None	Potassium cyanide (*M*)				CO_2 %
		0	10^{-5}	10^{-4}	10^{-3}	10^{-2}	50
40	A	46.5	58.6	69.7	71.6	60.5	48.0
	B	93.0	79.4	86.5	86.8	72.5	91.0
	C	46.5	20.8	16.8	15.2	12.0	43.0
50	A	55.5	69.9	83.2	85.5	72.2	59.0
	B	111.0	94.0	103.0	103.2	87.0	124.0
	C	55.5	24.2	19.8	17.7	14.8	65.0

A: Endogenous respiration, B: Acetate oxidation, C: Difference between A and B.

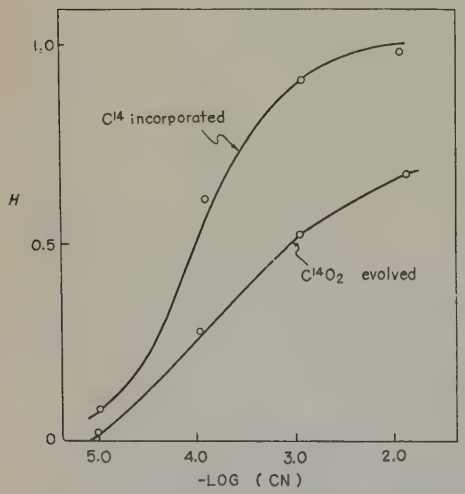

FIG. 5. Effect of cyanide on assimilation of carboxyl C^{14} and evolution of $C^{14}O_2$ (substrate : carboxyl labelled acetate).

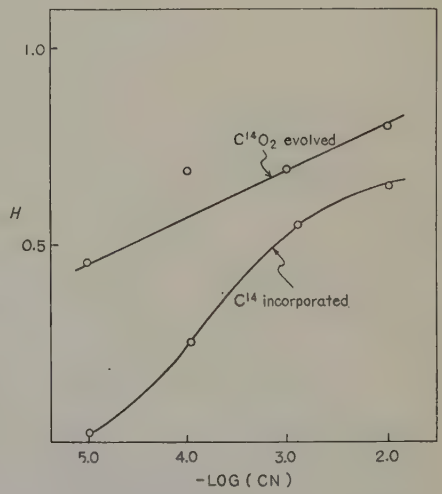

FIG. 6. Effect of cyanide on assimilation of methyl C^{14} and evolution of $C^{14}O_2$ (substrate : methyl labelled acetate).

nucleic acids and insoluble carbohydrates, contained about 17 per cent of incorporated carbon. It is remarkable with this particular fraction that a

portion as high as 80 per cent of its carbon was derived from the methyl carbon of the substrate.

Effects of Potassium Cyanide—In the previous study of this series we have shown that the addition of potassium cyanide in a concentration as low as 10^{-4} *M* effected a marked inhibition of O_2 uptake in the presence of acetic acid. One of the representative results of manometric experiments is illustrated in Fig. 4. From curve C (Fig. 4), which represents the acetic acid-induced surplus in O_2 uptake as affected by varied concentrations of potassium cyanide, the degree of inhibition H was computed as shown in Fig. 4 and Table III.

Results of isotope measurements under similar experimental conditions, with carboxyl- and methyl-labelled acetic acids as the substrate, are summarized in Tables IV and V. The degree of inhibition by cyanide was

TABLE IV

Cyanide Inhibition of C^{14}-Incorporation and $C^{14}O_2$-Evolution from $CH_3C^{14}OOH$

Temperature: 25°, pH: 7.0, Incubation time: 150 minutes.

Concentration of KCN (*M*)	0	10^{-5}	10^{-4}	10^{-3}	10^{-2}
Incorporated-C^{14}	4500	4280	1760	445	310
($C^{14}O_2$)	4386	4414	3194	2162	1482

(*c.p.m.*)

computed from these data and plotted in Figs. 5 and 6 as a function of cyanide concentration. In the case of the carboxyl carbon, the inhibition was found to be in accord with the formula of the first order sygmoid curve, with a value of the inhibition constant *K** of about 10^{-4} *M*. With methyl carbon, the assimilation was found to be more refractory to the action of the poison, only a partial inhibition by 60 per cent being effected even with a concentration of cyanide as high as 10^{-2} *M*. It will be noticed from comparison of these results that with a concentration of 10^{-5} *M* cyanide, with which there is an inhibition of acetate-induced O_2-uptake of about 50 per cent (see Fig. 4), there is no inhibition whatsoever of assimilation

* $$H=\frac{1}{1+K/(G)} \qquad (1)$$

Where (*G*) is the concentration of the poison, *K* is the constant of inhibition and *H* represents the grade of inhibition which will be defined as follows:

$$H=\frac{V-V_i}{V} \qquad (2)$$

in which V_i and V stand for the reaction rates in the presence and in the absence, respectively of the poison.

TABLE V

Cyanide Inhibition of C^{14}-Incorporation and $C^{14}O_2$-Evolution from $C^{14}H_3COOH$

Temperature: 25°, pH: 7.0, Incubation time: 50 minutes.

Concentration of KCN (M)	0	10^{-5}	10^{-4}	10^{-3}	10^{-2}
Incorporated-C^{14}	2225	2190	1820	1320	780
($C^{14}O_2$)	615	338	203	184	117

(*c.p.m.*)

with respect to either the methyl or the carboxyl carbon of the substrate. With higher concentrations of cyanide, for instance 10^{-2} M the assimilation from carboxyl carbon was almost completely suppressed, while the magnitude of methyl carbon assimilation was nearly the same as that of the O_2-uptake

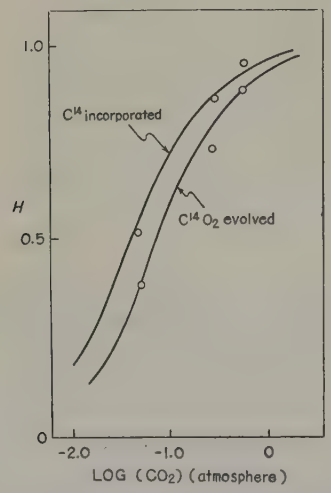

FIG. 7. Effect of CO_2-partial pressure on assimilation of carboxyl carbon and evolution of $C^{14}O_2$.

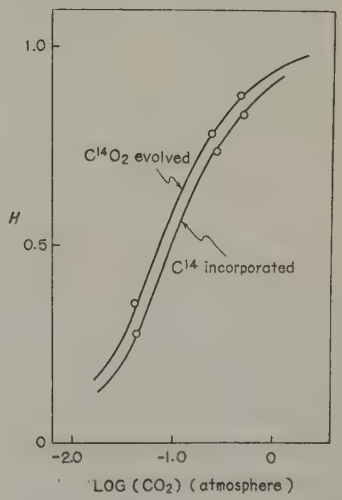

FIG. 8. Effect of CO_2-partial pressure on assimilation of methyl carbon and evolution of $C^{14}O_2$.

(about 60 per cent). In the isotope experiments we have also examined the cyanide inhibition of the CO_2-output. The result with carboxyl carbon gave rise to an S-shaped curve, which showed a striking coincidence with Curve C (Fig. 6), representing the cyanide inhibition of methyl incorporation. Quite different was the circumstance in the case of the inhibition of CO_2-output from methyl carbon as illustrated in curve D, Fig. 6.

Effect of Carbon Dioxide—In the course of isotope experiments it was discovered that the addition of carbon dioxide to the gas atmosphere caused

a striking depression of assimilation and decrease in the $C^{14}O_2$-output of *Chlorella* cells. Figs. 7 and 8 illustrate the experimental results obtained in this respect. In the case of assimilation and $C^{14}O_2$-output with methyl-labelled acetate as the substrate, 50 per cent inhibition was reached at partial pressure of about $10^{-1.0}$ atm. CO_2. The extent of inhibition with respect to $C^{14}O_2$-output from carboxyl carbon was the same as in the former cases we have seen in Fig. 8. Special care was taken to control the possible error due to the shift in hydrogen ion concentration of the medium. The pH-shift was tested by following the change passing a stream of air containing 30 or 50 per cent CO_2 through the reaction mixture. In both cases

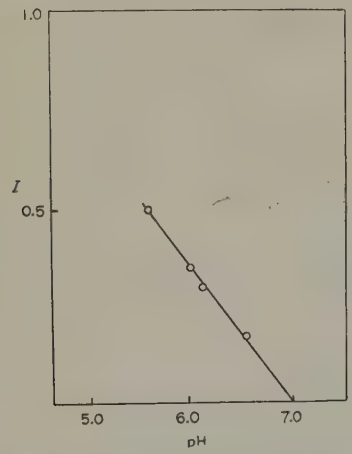

FIG. 9. Effect of pH on incorporation of $CH_3C^{14}OOH$. The grade of inhibition (I) was computed according to the following equation; $I=1-V/V_{pH_7}$, where V_{pH_7} and V stand respectively, reaction velocities at pH 7 and at the pH indicated.

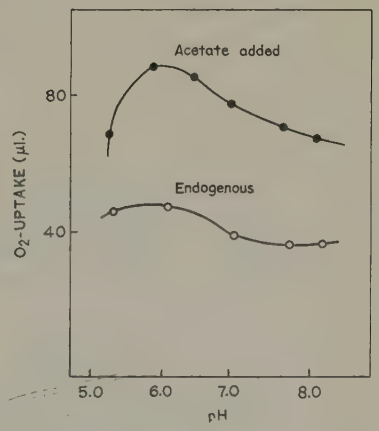

FIG. 10. Effect of pH on oxidation of acetate.

only a shift of 0.6 units of pH was discovered as an effect of CO_2. The shift, if even, of the medium as the cause of the CO_2-inhibition in question will safely be neglected in view of the experimental data on the pH-activity relationships shown in Figs. 9 and 10. Of course, the possible effect of selfshading in working with the use of a thicker layer of barium carbonate in CO_2-inhibition was controlled with the use of the standard curve for the correction of self-absorption, which was found to be satisfactorily reproducible.

The O_2-uptake of *Chlorella* cells, on the other hand, was little influenced by the addition of CO_2 to the gas atmosphere, as will be seen from the last column of Table III. Also the conversion of cellular carbon to CO_2 was shown to be unaffected by the addition of carbon dioxide to the gas atmosphere (Fig. 11). In this experiment, the endogenous output of radioactive CO_2 from C^{14}-labelled *Chlorella* cells was followed in ordinary air

and in CO_2-enriched air (50 per cent CO_2). The alteration in partial pressure of oxygen, which ranged from 20 per cent in the control to 10 per cent in the experiment with the highest CO_2 pressure studied (50 per cent CO_2), had no effect whatsoever on the metabolic processes under investigation.

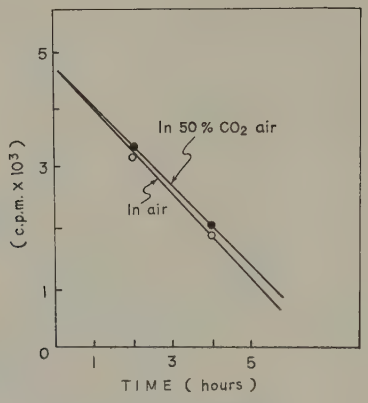

FIG. 11. Effect of CO_2 (unlabelled) on the decrease in C^{14}-level in cells during respiration.

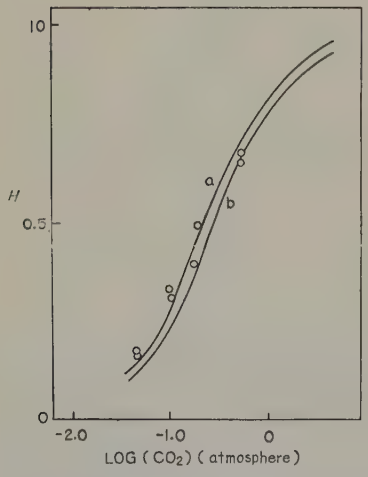

FIG. 12. Effect of CO_2 on assimilation of glucose and evolution of $C^{14}O_2$.

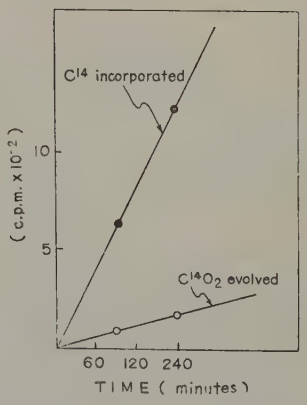

FIG. 13. Assimilation of glucose and evolution of $C^{14}O_2$ (substrate: all-labelled glucose).

The oxidative assimilation of glucose was also affected by the addition of CO_2 to the gas atmosphere (Fig. 12). Uniformly labelled glucose was used as the substrate, and the assimilation was estimated by measuring the total amount of radioactivity incorporated (Fig. 13). There was also an inhibition caused by CO_2. The intensity of inhibition of the glucose system

was, however, somewhat lower than in the case of acetate metabolism, 50 per cent inhibition being reached at a CO_2 partial pressue of $10^{-0.6}$. In this connection the assimilation and CO_2-output with glucose as the substrate, and the effect of the substance on the acetic acid metabolism were also followed. Table VI presents the results of these experiments. With a concentration of 10^{-2} *M* glucose, a 30 per cent inhibition of assimilation of acetate carbon (methyl) was noticed.

TABLE VI

Inhibitory Effect of Glucose (unlabelled) on the Incorporation of Acetate

(Methyl carbon; 10^{-2} *M*)

Glucose (*M*) / Time (*min.*)	0	10^{-3}	10^{-2}
20	344	—	216 (37% *inhibition*)
30	497	416 (7% *inhibition*)	364 (26.8% „)
40	750	800	500 (33% „)

(*c.p.m.*)

TABLE VII

Changes in Assimilatory Activity of Carboxyl Carbon of Acetate during Cellular Life-Cycle of Chlorella

Incubation time: 60 minutes.

Cell stage / Activity/V_p	D_n	D_a	L_1	L_2
C^{14}-incorporated	6270	10250	16850	18000
($C^{14}O_2$) evolved	2910	3220	4200	3440
($C^{14}H_2O$)/($C^{14}O_2$)	2.15	3.15	4.01	5.24

(*c.p.m.*)

D_n: "*Nascent dark cells*" which was used as the starting material of this experiment (average cell diameter: 2.61 μ), D_a: "*Active dark cells*" obtained after 6–8 hours of culture at 25° (average cell diameter: 3.17 μ), L_1: "*Light cells*" obtained after 13–15 hours of culture at 25° (average cell diameter: 4.81 μ), L_2: "*Lgiht cells*" obtained after 20–25 hours of culture at 25° (average cell diameter: 5.91 μ), V_p: Packed cell-volume.

Changes in Assimilatory Capacity during the Life-Cycle of Chlorella—Changes in the relative rates of oxidative assimilation and CO_2 output were followed,

using carboxyl-labelled aceti cacid as the substrate. Synchronized cells of *Chlorella* in various stages of the cellular life-cycle were used (see above, Tamiya *et al.* (*3*)). Starting from the stage of "nascent dark cells", both activities were found to increase along with the development of the cell in the course of life-cycle (Table VII). The rise in assimilation was, however, much more pronounced than the increase in CO_2-output, the ratio of the former to the latter activity thus showing a gradual increase from 2.15, in the initial stage, to 5.24 in "light cells". The above-stated changes were shown to be actually reproducible in that the "nascent dark cells" produced from the "light cells", showed the same level of metabolic activities as previously observed with the initial sample of "light cells".

The experiment was repeated, this time using methyl-labelled acetate

TABLE VIII

Assimilation of Methyl Carbon of Acetate in Various Stages of Life-Cycle of Chlorella

Incubation time: 30 minutes.

V_p: Packed cell volume.

Activity/V_p \ Cell stage	D_n	D_a	L_1	L_2
C^{14}-incorporated	11100	13600	14100	9300
($C^{14}O_2$) evolved	0	0	0	0

(*c.p.m.*)

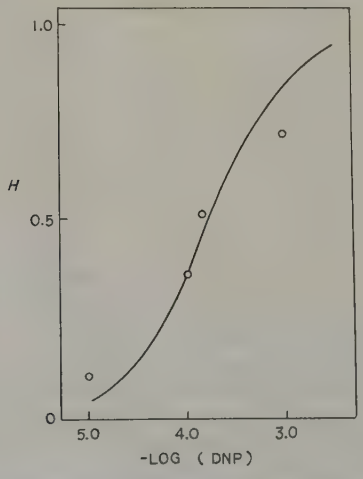

FIG. 14. Effect of 2,4-dinitrophenol on assimilation of $CH_3C^{14}OOH$.

as the substrate (Table VIII). In this case, there was no appreciable change in the incorporation rate. An immediate evolution of $C^{14}O_2$ from the methyl carbon was not observed in any stage of cellular life-cycle, there being always a lag period of 30 to 40 minutes before the appearance of radio carbon as carbon dioxide.

Effect of 2,4-Dinitrophenol—In the foregoing work 2,4-dinitrophenol, in a concentration of 10^{-3} *M* has been investigated to effect an 80 per cent inhibition of the respiratory oxidation of acetate under similar experimental conditions. The assimilation of acetate was found to be equally sensitive to 2,4-dinitrophenol (Fig. 14), 50 per cent inhibition being observed with a concentration of the poison as low as $10^{-3.8}$ *M*. In this experiment, carboxyl labelled acetate was used as the substrate.

DISCUSSION

In the cultivation in the dark, acetate and glucose are utilized by *Chlorella* cells as the organic source of carbon favorably supporting unlimited growth. All the normal ingredients, including chlorophyll, are synthesized from acetic acid.

The most salient feature of the results obtained in this study is the markedly high efficiency with which these metabolites are incorporated in the cells of *Chlorella*. The above-mentioned values for the efficiency of assimilation of about 1.4 atoms carbon incorporated per one molecule uptake of O_2 are perhaps among the highest thus far reported in this respect (see Clifton (*4*)). The high proportion of C^{14} utilized as compared with C^{14} discharged as carbon dioxide is another expression of the same fact. The outstanding capacity of *Chlorella* for the efficient utilization of acetic acid (and glucose) has also been noted in the works of Clifton, who has been working with the ordinary test material of the organism obtained by the usual method of cultivation. Through the use of the synchronous culture technique we have elucidated the circumstance that the high efficiency of utilization is especially marked in the "Light cell" stage of their life cycle, although the rates of both carbon dioxide evolution and incorporation show gradual increase along the progress of the life-cycle from the *dark* to the *light* cell stage. Cells of *Chlorella* contain considerable amounts of glucose and conceivably a certain amount of acetic acid as the normal ingredients of the cell, although neither of these substance is involved in the list of immediate intermediates of photosynthesis. The conversion of these substances into various cell material must be taking place in the normal life of the cell. Our finding that light had no effect whatsoever on the rate and efficiency of acetate assimilation (see above) indicates that there is no interaction between the two processes. Also Myers (*5*) has similarly reported a lack of effect of light on the oxidative assimilation of glucose in the case of *Chlorella pyrenoidosa*.

The different behaviors of the methyl and carboxyl moieties of acetic acid in the assimilation suggest the transfer mainly of the methyl group of

the acid into cell substance. As to the precise nature of the intermediate to or through which this methyl carbon is transferred, no substantial evidence has yet been obtained. The increasing discharge of methyl carbon in the carbon dioxide fraction in later periods of incubation (see Table I) is most plausibly accounted for by assuming a secondary oxidation and/or decarboxylation of the accumulating products of assimilation that had been primarily formed from methyl carbon. As shown by the experimental results, a relatively small portion of the organic material is derived from the carboxyl carbon of acetic acid. The relative insensitivity of the main assimilatory process (*i.e.* the path from methyl carbon) as compared with that of the subsidiary path (*i.e.* the path from carboxyl carbon) as well as the oxygen uptake, should perhaps be noted.

The discovery of an inhibitory action of carbon dioxide on the assimilation process and the CO_2 output deserves special consideration. The possible intervention here of an isotopic dilution effect is to be excluded, since there is evidence that in the process of assimilation, free carbon dioxide was not playing the part of intermediary of the assimilatory processes under investigation. In the first place, the rates of incorporation of acetic acid with respect to either methyl or carboxyl carbon are not influenced by the presence or absence of the absorbent, KOH, in the center well of the reaction vessel. Secondly, the rate of dark-fixation of labelled carbon dioxide under similar experimental conditions of the organism is far less (about 1/20–1/40 according to the results in Table IX) than those of the assimilatory process(es)

TABLE IX

Dark CO_2-Fixation and Methyl Carbon Incorporation of Acetate by Chlorella Cells

Temperature: 25.0°, pH: 7.0.

Carbon *mM/ml.* cell / Time (*min.*)	Carbon incorporated from acetate (a)	Dark CO_2-fixation		b/a	c/a
		(b) Without acetate	(c) With acetate		
10	1.58×10^{-4}	10.1×10^{-6}	6.1×10^{-6}	1/15.6	1/25.7
20	3.00×10^{-4}	16.7×10^{-6}	15.2×10^{-6}	1/18.0	1/19.7
30	6.70×10^{-4}	26.6×10^{-6}	17.4×10^{-6}	1/22.5	1/38.5

here in question. Thirdly, the results of the tests by the present author showed that the rate of this dark-fixation of $C^{14}O_2$ was not altered in magnitude by the presence or absence of (unlabelled) acetic acid in the reaction mixture. This again excludes the possibility that CO_2 was incorporated with significant rapidity; even in the presence of acetic acid as the substrate of assimilation. The only way of accounting for the finding is to

assume that carbon dioxide must be involved as an actual inhibitor of assimilatory process(es).

Such mutual independence of photosynthetic and assimilatory processes is further evidenced by the fact that the CO_2 inhibition of the incorporation is little influenced by illumination, a condition under which free carbon dioxide is readily assimilated photosynthetically.

The investigation of the precise mechanism as to the actual pathway of acetic acid in the assimilatory process is being continued.

SUMMARY

1. The process of oxidative assimilation in the green alga, *Chlorella ellipsoidea*, was investigated, using methyl- and carboxyl-labelled acetic acids and glucose as the substrates.

2. It was found that the rate as well as the balance sheet of the oxidative assimilation of carboxyl carbon of acetic acid was subject to considerable change in the course of cellular life-cycle of the organism. No significant change with this respect was observed with methyl carbon as the substrate.

3. The efficiency of acetic acid incorporation was found to be very high, in the most favorable cases, 1.6 atoms carbon being assimilated per one molecule acetic acid consumed.

4. The conversion of the methyl and carboxyl carbon of acetic acid was investigated by isotope experiments. The methyl carbon was found to be converted exclusively into cell material; while two-thirds of the carboxyl carbon was found to be incorporated in cell material. The remaining one-third of the carboxyl carbon was discharged as carbon dioxide.

5. The incorporation of the carboxyl carbon was inhibited by cyanide, and according to the first order sygmoid curve (see text); 50 per cent inhibition being reached at $10^{-4.0}$ *M* potassium cyanide; the incorporation from the methyl carbon and $C^{14}O_2$ evolution from the carboxyl carbon were found to be more refractory to the poison, 50 per cent inhibition being reached at $10^{-3.0}$ *M* of potassium cyanide. No complete inhibition of the incorporation of methyl carbon and the $C^{14}O_2$-evolution from carboxyl carbon was observed at a concentration of potassium cyanide as high as 10^{-2} *M*.

6. Carbon dioxide (unlabelled) was found to inhibit all the metabolic processes in accord with the first order sygmoid curve; partial pressure of carbon dioxide for 50 per cent inhibition of the incorporation from the carboxyl, and from the methyl carbon, and CO_2 evolution from the carboxyl and methyl carbon were found to be $10^{-1.5}$, $10^{-1.0}$, $10^{-1.0}$, $10^{-1.0}$ atom, respectively.

7. 2,4-dinitrophenol was found to inhibit the incorporation of carboxyl carbon into the organism.

I wish to express my thanks to Prof. H. Tamiya for suggesting this investigation

as well as for constant guidance in the course of the work. Thanks are also due to Prof. A. Takamiya for some valuable suggestions.

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STUDIES ON THE RESPIRATION OF THE TOOTH GERM

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It has been recognized by both early and recent investigators of the mechanism of the calcification that the glycolytic process of the Embden-Meyerhof-Parnas scheme occurs in calcifying tissues, in general (*1–4*). In 1950, Gutman and Yu (*5*) found the significant fact that phlorizin, monoiodoacetate, and fluoride, the well-known inhibitors of glycolysis, decreased the calcification rate of rachitic cartilage *in vitro*, and based on this fact, they postulated that the glycolytic process is directly related to the calcification mechanism. That is, the energy-rich phosphate in phosphopyruvate derived from glycolysis may be transphosphorylated to the matrix in which mineralization occurs.

On the other hand, while the presence of a large amount of citrate in the calcified tissue has been known for a long time (*6*), Dixon and Perkins (*7*), and recently Less and Kuyper (*8*), found the presence of the enzymes and intermediates concerned in the TCA cycle. Tulpule and Patwardhan (*9*) discovered that vitamin D increased the pyruvate oxidation which had previously decreased in the case of rachitic metaphysis. In addition, it was found that respiration was necessary for the biosynthesis of chondroitin sulfate, which is considered to be an important portion of the organic matrix for calcification (*10*). Thus the respiratory system has become another important subject of research because of its possibly important role in process of calcification, particulary in the period of matrix formation.

These experiments were performed on the tissues other than tooth such as cartilage (preosseous or rachitic), bone, callus after fracture, *etc.*

The biological activities of the tooth after it is completely formed are very slight, so that there is only a small probability for the tooth to recover when it once get caries. However, the tooth germs have the most active calcifying ability and carry out intense calcification in a relatively short period. Also the metabolism responsible for tooth formation might be different from that of other hard tissues.

In order to gain a better insight into the biochemistry of calcification metabolism, especially in the case of the tooth, the author intends, in the present study, to investigate the respiratory activity of tooth germs. In his previous research, Pincus (*11*) found the presence of respiration in human

tooth pulp, and that the mean Q_{O_2} value was 0.31. Flieder and Fisher (*12*) found a Q_{O_2} value of 0.56 for bovine tooth pulp. However, it may be supposed that these pulp tissues were not directly concerned in calcification, because they did not contain odontoblasts and the teeth were almost completely calcified.

EXPERIMENTALS

Removal of the Tooth Germ—The dogs about 1 or 2 months after birth were sacrificed. Tooth germs of the following permanent teeth were selected for the experiment because they are of sufficient size to be studied: The fourth premolar, first upper molar, and first lower molar. After removing the jaw from the body, these germs were extracted by breaking the alveolar bone with care to avoid injury of the dental sac. X-ray photographs were taken before the slaughter in order to record the degree of calcification which would indicate the age of the dog. The body weights of these animals were also determined previously.

Separation into Three Tissue Parts—Each of three tooth germs was weighed by torsion balance and, after being kept in cold saline solution, separated with tweezers into the following three parts:

The first tissue part was the dental sac and the enamel pulp (stellate reticulum), which is present between the dental sac and the calcified part.

The second part, the calcified part, consisting of immature dentine and enamel, was removed from the dental papilla, which was located inside the calcified part.

The third part was the dental papilla.

Each of these parts was individually weighed and put into the respective Warburg vessels which contained Krebs-Ringer's buffer solution and was kept in ice-cold water. The dental sac and the enamel pulp part, and the calcified part were used directly for the experiment, but the papilla part was used as the slice which was chopped by a hand slicer to about 0.5 mm. of thickness. From the time the tooth germs were removed until the beginning of the manometric procedure, the tissue materials were kept cold throughout, in order that they might maintain biological activity.

It would be desirable to determine the respiration of three tissue parts for each of the tooth germs, but in order to get a sufficient amount of tissue for manometry, the dental sac and the enamel pulp taken from the three tooth germs were placed in one vessel, the calcified part from the three tooth germs were placed in another and the dental papilla in another. One group of the vessels containing the three tissue parts from the right jaw and one group containing those from the left jaw were used. One group was used to detect the effect of the substrates or other substances and another for the control experiment.

Composition of the Reaction Mixture in the Vessels—In general, the following composition of the mixture was used. In the main compartment of the vessel each separated tissue part was suspended in 1.6 ml. of Krebs-Ringer's phosphate buffer. In the center well, 0.2 ml. of 20 per cent KOH solution was placed with a piece of filter paper. In the sidearm, for one group of vessels 0.2 ml. of neutralized substrate or other solution was placed, and for another control group 0.2 ml. of Krebs-Ringer's solution was placed.

Condition of Warburg Manometric Techniques—The gas phase of the vessels was filled with oxygen after pumping off the air. After the preincubation of 10–15 minutes at 37°, the oxygen uptake was read during 2 hours. The substrate solution or Krebs-Ringer's buffer solution in the sidearm were tipped into the main compartment of the vessel at 30

minutes or 1 hour after the initial reading. The rate of respiration was compared before and after the addition of the substrate.

Determination of Dry Tissue Weight—After the manometric procedure, 2 ml. of 7 per cent formaldehyde solution was added to each vessel and then the contents of the vessels except the KOH solution were transferred into sintered glass filters for the purpose of collecting the tissue materials, which were then washed with water. It took 2 hours to dry the tissues on the glass filters by heating at 100–105°. The weight of the dried tissue was obtained from the difference between the weight of the glass filter with and without that tissue.

Determination of Respiratory Quotient (*RQ*)—According to Dickens-Simer's first method (*13*), the author measured respiratory quotient with vessels having a Siamese sidearm. In the first sidearm 2 *N* KOH was placed with a piece of filter paper, and in the second, 4 *N* H_2SO_4 solution. Two groups of the vessels were used, one for determination of O_2-uptake and thereafter for CO_2 production, and the other for the determination of CO_2 which was endogeneously present in the tissues and solutions.

Determination of the Degree of Calcification in Terms of "Calcification Coefficient"—In order to express quantitatively the degree of calcification, the author used the "calcification coefficient", which is the ratio of the dry weight of the calcified parts, to the whole dry weight of the tooth germs. This value is zero when the calcification has not yet occurred, and approaches 1.0 with the progress of calcification.

Determination of Nitrogen—For the calculation of $Q_{O_2}(N)$, the nitrogen content of the tissue parts was determined by the micro-Kjeldahl-Parnas method.

RESULTS

Endogeneous Respiration—The O_2-uptake by the three tissue parts without any addition of substrate occurred almost linearly for about 2 hours. The data concerning the rate of the endogeneous respirations are collected in

TABLE I

Rate of Respiration of the Three Tissue Parts

Tissue part	Q_{O_2}		$Q_{O_2}(N)$	
	Mean	No. of experiments	Mean	No. of experiments
Enamel pulp and dental sac	2.63 (0.76)	19	17.0 (4.2)	4
Dental papilla	3.56 (1.07)	18	25.3 (5.8)	4
Calcified part			1.1 (0.3)	4
Calculated by assumed dry weight	6.38 (4.27)	14		
Calculated by EDTA decalcification	0.72 (0.13)	6		

Standard deviations are given in bracket.

Table I as Q_{O_2} (O_2-uptake μl./hours/mg. dried tissue). These calculations

were done for the enamel pulp including the dental sac part and the dental papilla by using the dry weight which was obtained by the above-mentioned method. However, as for the calcified part, its conditions are very different because this tissue part has a large amount of inorganic substance, *i.e.* enamel and dentine which have no activity in respiration. Thus the Q_{O_2} value of the calcified part, obtained directly by using the dry weight, cannot be compared to that of other tissue parts. For the comparison, therefore, Q_{O_2} value of the calcified part was calculated by using an assumed dry weight of active soft tissue, and also by the dry weight determined after the demineralization with ethylenediamine tetraacetate. In addition, $Q_{O_2}(N)$ values (O_2-uptake μl./hour/mg. nitrogen content of the tissue) were determined for the three tissue parts.

In the first determination, an assumed dry weight of the soft tissue on the calcified part was calculated. It is supposed that the soft tissue which might be present on the calcified part is responsible for the respiration, and the Q_{O_2} value must be calculated on the basis of dry weight of this soft tissue. The details of this supposition will be discussed later, the approximate value of this weight was calculated as follows: The ratio of decrease in weight caused by drying was different for the three tissues. The mean ratio of dry to fresh weight was 1/19.3 for enamel pulp including dental sac part, and 1/23.3 for dental papilla, while for calcified part this value was 1/1.36. Therefore, under the assumption that this decrease would be due to the soft tissue element on the surface of the calcified part and that the weight of inorganic tissue elements would not change by drying, the assumed weight of the dried soft tissue element was obtained by the equations:

$$21x+y=a \quad \text{..... fresh condition (I)}$$
$$x+y=b \quad \text{..... dry condition (II)}$$

where,

a=fresh weight of the calcified part
b=dry weight of the calcified part
x=dry weight of the soft tissue element
y=weight of inorganic tissue element

therefore from the equation (I) and (II)

$$x=\frac{a-b}{20}$$

The Q_{O_2} for calcified part was calculated using the value x as the dry weight.

In the second determination, Q_{O_2} values were determined by using the dry weight after decalcification with 10 per cent ethylendiamine tetraacetic acid (EDTA) solution.

The values of $Q_{O_2}(N)$ for the three tissue parts are also shown in Table I.

The Q_{O_2} values for enamel pulp including dental sac part were between 1.5 and 4.0 and the mean value was 2.63 ± 0.76, and that for dental papilla was somewhat higher, between 2.0 and 4.8 and the mean was 3.56 ± 1.07. For calcified part, the values which were calculated on the basis of above-

mentioned assumed weight, showed a marked fluctuation. The source of this fluctuation was due in part to the error in manometer reading caused by the smallness of the absolute amount of O_2-uptake, but the degree of calcification plays a role in the fluctuation. The mean value of Q_{O_2} for the calcified part which is not fully reliable because of the fluctuation, was 6.38 ± 4.27. This is higher than that for either of the other tissue parts. However, in six cases, whose Q_{O_2} were calculated by the dry weight after the demineralization with EDTA, the results indicated considerably lower activities and the deviation was smaller (0.72 ± 0.13). In addition, comparing $Q_{O_2}(N)$ of three tissue parts the value for the calcified part was 1.1 ± 0.3, and was also lower than that for the enamel pulp or the dental papilla.

The Correlation in Endogeneous Q_{O_2} and the Degree of Calcification—The values of Q_{O_2} were plotted against the degree of calcification. The results are shown in Figs. 1, 2 and 3. In the enamel pulp including the dental sac part (Fig. 1) and the dental papilla (Fig. 2), the trend was found that with the progress of the calcification the rate of respiratory activity increased, on the contrary in the calcified part (Fig. 3) the Q_{O_2} values calculated by assumed dry weight, the trend was to decrease. The correlation coefficients were 0.43 for the enamel pulp including the dental sac part, 0.46 for the dental papilla and -0.55 for the calcified part.

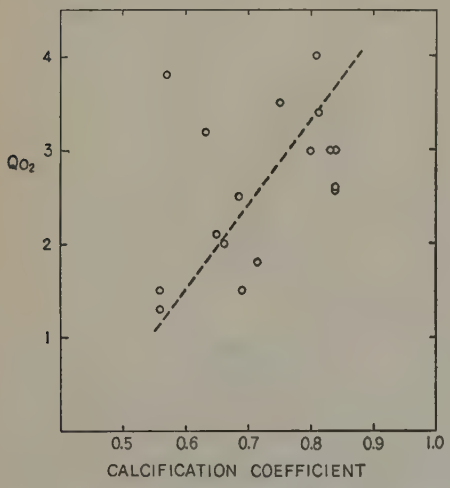

FIG. 1. Correlation between Q_{O_2} values and "calcification coefficient" for the enamel pulp including the dental sac part. Correlation coefficient was 0.43.

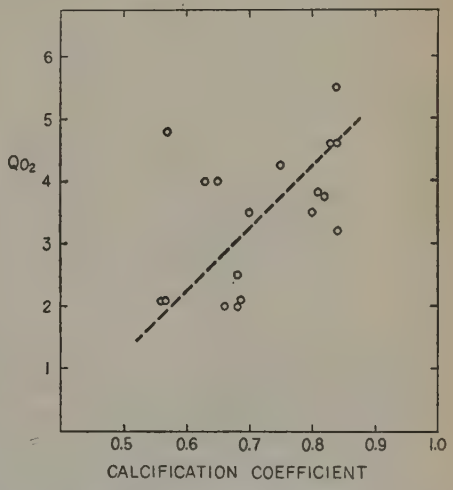

FIG. 2. Correlation between Q_{O_2} values and "calcification coefficient" for the dental papilla. Correlation coefficient was 0.46.

The Effect of Substrate Addition—The neutralized solution of substrates concerned in glycolysis or TCA cycle were added the tissues in endogeneous respiration and the change of the rate in O_2-uptake was observed. Glucose, lactate, pyruvate, citrate, α-ketoglutarate, succinate, fumarate, malate, and

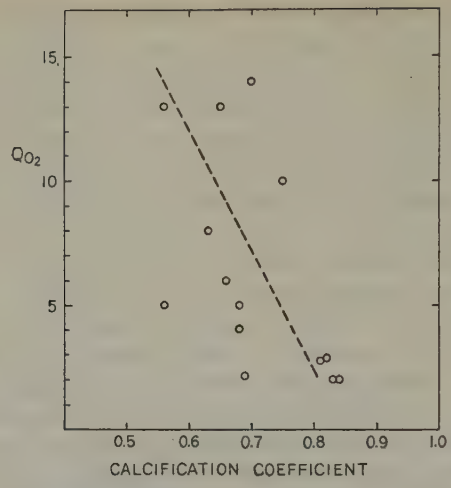

FIG. 3. Correlation between Q_{O_2} values and "calcification coefficient" for the calcified part. Correlation coefficient was -0.55. Q_{O_2} values were calculated by assumed dry weight.

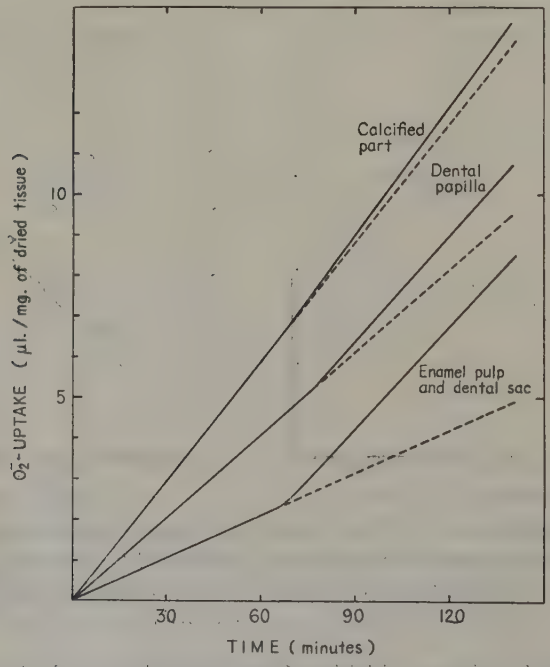

FIG. 4. Effect of α-ketoglutarate addition. 0.01 M α-ketoglutarate solution was added at the 60th minute. Control experiments are shown as broken line. In the case of the calcified part, the O_2-uptake values were calculated by assumed dry weight.

glutamate were studied as substrates. As shown in Fig. 4 with the addition of 0.01 *M* α-ketoglutarate, the O_2-uptake increased in all three tissue parts. The increase in O_2-uptake with α-ketoglutarate was always observed, but the rate of increase was not constant. The other substrates had no effect on the respiratory rate except one case in which fumarate promoted considerable increase in O_2-uptake for the dental papilla.

The Effect of Cytochrome c and Methylene Blue—The addition of 6×10^{-4} *M* cytochrome c (from muscle, L. Light & Co., Ltd.) was carried out in the same manner as in the experiment of substrate addition. The results are presented in Fig. 5, and the results of methylene blue (3×10^{-4} *M*) are given in Fig. 6. In both cases the increase in O_2-uptake was definite.

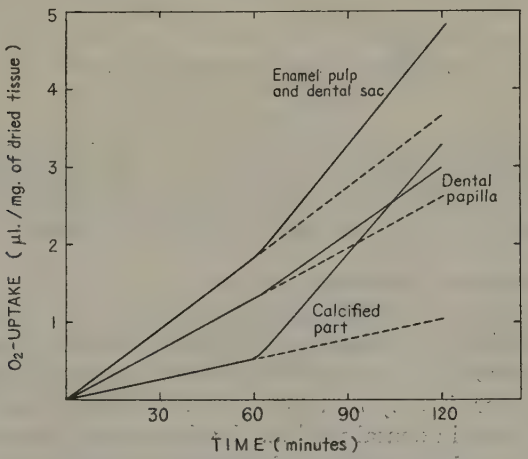

FIG. 5. Effect of cytochrome c addition. 6×10^{-4} *M* cytochrome c was added at the 60th minute. Control experiments are shown as broken lines. In the case of the calcified part, the O_2-uptake values were calculated by assumed dry weight.

The Effect of Removing Calcium Ion—The respiration was determined by suspending the tissues in a buffer solution, which had the same constitution as Krebs-Ringer's solution except it had no $CaCl_2$, in order to investigate the influence of calcium ion on the respiration. The author supposed that there may be some relationship between calcium metabolism and respiratory activity. The values of Q_{O_2} were not different with and without calcium. Furthermore, EDTA solution (final concentration 10^{-2} *M*, pH 7.4) was added to respirating tissues for the purpose of removing calcium which had been endogeneously present in the tissue. However, the rate was still unchanged. In one case, from the addition of EDTA, the calcified part manifested a considerable increase in respiratory activity, but in the other two cases, such a phenomenon was not observed. The meaning of this increase is not clear; however, one possibility is that the mineral elements which had partially enclosed the cells were removed and that the respiration

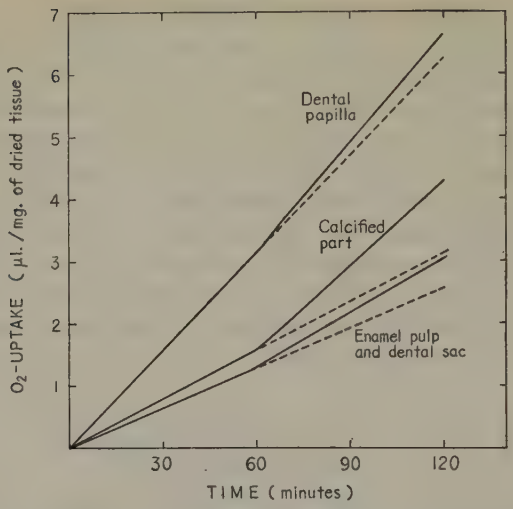

FIG. 6. Effect of methylene blue addition. $3 \times 10^{-4} M$ methylene blue was added at the 60th minute. Control experiments are shown as broken lines. In the case of the calcified part, the O_2-uptake values were calculated by assumed dry weight.

then appeared in its full activity.

Respiratory Quotient—The RQ determined for dental papilla were 0.96, 0.98, 1.10, indicating that the quotient was near 1.0. For enamel pulp including dental sac part, the results were not consistent, such as 0.15, 0.17, 0.46, 0.66. However, it may be mentioned that in this tissue the value was somewhat lower than in dental papilla. In the case of the calcified part, the RQ value could not be determined because the tissue had a large amount of carbonic salts, which made it difficult to determine the accurate amount of CO_2 produced by respiration.

DISCUSSION

Q_{O_2} values obtained in this experiment were 2.63 for the enamel pulp and the dental sac part and 3.56 for the dental papilla. These values are considerably higher compared to those of other tissues. This activity is much higher than that of cartilage, which was 0.001–0.01 (*3*, *4*), or of skin which was 1.2–1.3 (*14*), or of dental pulp of the human tooth which was 0.31 (*11*) or of the bovine tooth which was 0.56 (*12*), and about the same as that of gingiva which was 1.6–2.0 (*15*).

In order to know what kind of histological elements are present in the three tissue parts which were separated in the above-mentioned manner for manometric studies, each separated part was prepared as a histological specimen, after the decalcification with formic acid in the case of the calcified part. Then the microscopic examination was carried out. In the enamel pulp including the dental sac part, loose stellate reticulum cells were

found abundantly and among them ameloblasts forming in a line were present sparsely. In the dental papilla part, the surface which had faced to dentine was covered almost regularly by a layer of odontoblasts. On the other hand, on the surface of the calcified part no cellular component containing nuclei was found for the enamel and also the dentinal side. From these findings, the author judged that by the procedure for separation of the tooth germ into the three tissue parts, both ameloblasts and odontoblasts had been torn off from the pre-enamel and the pre-dentine surfaces. Pincus (*11*) reported that when human tooth pulp was pulled out, the odontoblast layer remained on the inner surface of the pulp cavity of the tooth. Concerning the discrepancy in the results between the present research and that of Pincus (*11*) as to which part the odontoblast attach, it is the author's opinion that in the case of the tooth germ, the union between odontoblasts and pre-dentine is not tight enough to hold the odontoblasts on the surface of pre-dentine, and that with the progress of the calcification, odontoblasts come to adhere to the dentine.

As indicated by the histological findings, no cellular matter was found on the surface of the calcified part, however respiration of the calcified part was always found, although the rate was not constant. Concerning this problem, the following two possibilities may be suggested: that, beyond the scope of the histological preparation, the cells were present on some part of detine or enamel surface, or that, torn cellular fragments or dentinal processes without nuclei manifested respiration.

The Q_{O_2} value of the calcified part obtained by assumed dry weight was higher than that of the other two parts, but the value by EDTA decalcification was lower and the $Q_{O_2}(N)$ was also lower than those of the other two parts.

An interesting finding is the relation between Q_{O_2} and the degree of calcification. The Q_{O_2} values for enamel pulp including the dental sac part and the dental papilla showed a trend to increase with the progress of calcification within the period examined, whereas, that for calcified part had a tendency to decrease. This fact suggests that in the course of the development of the tooth germs, the peak of oxidative metabolism is present at different stages for different tissues. Another remarkable result was that only α-ketoglutarate increased the O_2-uptake. Thus this substrate may play an essential role in the metabolic pathway, but may not be through glutamate because glutamate did not cause the increase in O_2-uptake. Methylene blue and cytochrome c increased the O_2-uptake. Tulpule and Patwardhan (*9*) found the same, in the experiment on cartilage slices. This phenomenon may be a result of these two substances aiding the electron transferring activity. There is a question about the permeability of the cell membrane to these substances; however, it may be a possibility that the separation procedure had ruptured the cell membrane.

The experimental results were almost the same for the three tissue parts investigated but the RQ values were different for the dental papilla and

for the enamel pulp including the dental sac part. The details of endogeneous respiration may be different.

SUMMARY

1. The tooth germs of the dog 1 or 2 months after birth were separated into three tissue parts: The enamel pulp including the dental sac, the dental papilla and the calcified part, the their respiratory activities were determined by the Warburg manometric technique. The mean Q_{O_2} values were 2.63 ± 0.76 for the enamel pulp including the dental sac part, 3.56 ± 1.07 for the dental papilla and for the calcified part an accurate value could not be determined, although respiration was always observed. The question as to what kind of cell component was responsible for the respiration of the calcified part could not be clarified even by means of histological research.

2. α-Ketoglutarate had the effect of increasing O_2-uptake in all three tissue parts. Other substrates: glucose, lactate, pyruvate, citrate, succinate, fumarate, malate, and glutamate had no effect.

3. Methylene blue (3×10^{-4} *M*) and cytochrome c (6×10^{-4} *M*) increased O_2-uptake in all three parts.

4. Removing the calcium ion from the medium had no effect on the rate of endogeneous respiration of the tissues.

5. From the results of the correlation in respiration to the degree of calcification, it was suggested that the maximum respiratory activity for each tissue part occurs at different stages of germ development.

6. The RQ value was about 1.0 for dental papilla, and for the enamel pulp including the dental sac part, a definite value was not obtained but was supposed to be smaller than that for dental papilla.

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THE EFFECT OF METAPERIODATE ON COMPLEMENT

A METHOD FOR PREPARING THE FOURTH COMPONENT OF COMPLEMENT

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In the course of the study on the mechanism of species difference in bringing about agglutination of kieselguhr by serum (*1*) the question was raised that metaperiodate might inactivate complement activity. The investigation revealed that metaperiodate inactivated three complement components C_1', C_2' and C_3' and spared selectively the fourth component C_4'. In this respect metaperiodate acts on complement just reversely to ammonia or hydrozine. The present paper reports the experimental observations and it seems that this finding would be an additional method for obtaining selectively the fourth component of complement.

METHODS

Sheep blood was collected in an Erlenmeyer flask containing an equal volume of Alsever's solution (*2*) and was mixed well. After filtering through cotton gauze the mixture was added with 400,000 units of crystalline penicillin G per 100 ml. of the filtered blood as a preservative according to Leon *et al.* (*3*) and stored in a refrigerator at 4°. The mixture was allowed to keep more than 24 hours (*4*) before use.

Veronal-bicarbonate buffer containing 0.00015 *M* $CaCl_2$ and 0.0005 *M* $MgCl_2$, pH 7.3 to 7.4 (which is referred to the following text as V. b.) (*5*) was used for washing the above sheep erythrocytes and for dilution of the erythrocytes and hemolysin except where especially noted.

Preparation of Sensitized Sheep Erythrocytes (EA') Suspension—An appropriate amount of sheep erythrocytes suspension was washed three times with large volume of V. b. by centrifugation and once more suspended in V. b. This suspension was centrifuged for 20 minutes at 2,500 r.p.m. and the supernatant was carefully siphoned off. Thus obtained packed erythrocytes were diluted to 1 : 5 with 4 volumes of V. b. To this 5-fold diluted erythrocytes suspension was added equal volume of 1 : 400 diluted hemolysin, *i.e.* twice the amount of the hemolysin necessary for the complete hemolysis of the erythrocytes used and it was allowed to stand at room temperature for 30 minutes (EA'). Preliminary experiment proved that the largest dilution of hemolysin which would cause complete hemolysis of the erythrocytes under the condition of the mixture of each equal volume of hemolysin, erythrocytes, and 1 : 10 diluted complement was 1 : 800. Hemolysin was obtained from rabbits which were five times, every fifth day, injected intravenously with five ml. each of 10 per cent sheep erythrocyte suspension on the twenty-third day from the first injection. The final concentration of these sensitized erythrocytes was, therefore,

approximately 1×10^9 erythrocytes per ml. (10 volume per cent) and optical density of this hemolysed solution gave 0.82 to 0.79 when measured with its 1 : 20 diluted solution with water at wave length of 541 mμ using 10 mm. thick cuvette.

Preparation of Reagents for Complement Component—Endpiece (R_1) as reagent for C_1' titration (C_1' designates the first component of complement according to the terminology proposed by Pillemer and Ecker (*6*), and so on ; C′ designates fresh serum or whole complement) was prepared by bubbling CO_2 gas for 10 minutes through 10 ml. of 1 : 10 diluted guinea pig serum (C′) with distilled water under the ice-cold temperature (*7*). The supernatant was separated from the sediment by centrifugation, neutralized by the addition of 0.1 *N* NaOH and made isotonic with 10 per cent NaCl solution. This solution was considered 1 : 10 diluted end-piece (R_1) and desired dilution was made by further addition of V. b., say 1 : 16 diluted end-piece (R_1) was made by adjusting the volume to 16 ml. with V. b. The sediment from the above procedure was washed three times wtih distilled water and dissolved in a desired amount of V. b., for example, if dissolved in 16 ml. the resulting solution is 1 : 16 diluted mid-piece (R_2). One ml. of C′ was treated with 2.0 ml. of 20 per cent baker's yeast suspension for 1.5 hours at 37° (*8*). This mixture was then centrifuged and the supernatant was diluted to desired volume with V. b. This yeast-treated serum (R_3) was considered the reagent for titration of C_3'. Reagent for the fourth component (R_4) was made by addition of 0.25 ml. of 0.15 *N* NH_4OH to one ml. of C′. This mixture was incubated at 37° for 1.5 hours and then neutralized by addition of 0.25 ml. of 0.15 *N* HCl (*9*) and diluted appropriately with V. b. as above. This ammonia-treated serum (R_4) was considered the reagent for C_4'. Heat-inactivated serum was prepared by heating at 56° for 30 minutes and diluted appropriately with V. b. as above.

Treatment of C′ with Sodium Metaperiodata (*$NaIO_4$*)—Undiluted or appropriately diluted C′ was added with 0.02 *M* $NaIO_4$ in the various ratios of volume (usually 1 : 1). This mixture was occasionally shaken and allowed to stand at room temperature for a definite length of time during which time the reaction proceeded. The reaction was stopped at desired time by addition of a certain amount of 0.449–0.225 *M* ethylene glycol solution, which decomposed the excess of $NaIO_4$ remaining in the mixture and at the same time made the resulting solution to be of the desired concentration in C′ For the preparation of sodium metaperiodate and ethylene glycol solutions and for the dilution of C′ for the present purpose 0.9 per cent NaCl solution, not V. b., was used throughout as $NaIO_4$ forms, though it was slow in reaction, insoluble product with V. b. This treatment was always carried out at room temperature.

Quantitative Titration of Complement Activity—According to Plescia *et al.* (*10*) when the concentration and volume of sensitized erythrocytes suspension and the volume of complement added are kept constant, logarithm of the half time ($t_{\frac{1}{2}}$), *i.e.*, the time required for the hemolysis to go half way to completion and the logarithm of the concentration of complement shows a linear relationship. Since $t_{\frac{1}{2}}$ can easily be determined experimentally one can estimate quantitatively the concentration or activity of complement used. For obtaining $t_{\frac{1}{2}}$ we followed a slight modification of a method developed by Mayer *et al.* (*5*). To 10 ml. of sensitized erythrocytes suspension 1 ml. of C′ was added and mixed by shaking and the tube was immediately immersed in a water bath of 37±0.2°. Both reactants were previously kept in ice-cold water. During this incubation at 37° for 1.5 hours, the tube was shaken periodically and six portions each of 1.5 ml. of the content were transferred into the tubes containing 4.5 ml. of ice-cold solution containing 0.12 *M* NaCl and 0.015 *M* sodium citrate. By this procedure progress of hemolysis was stopped at any desired stage, and after centrifugation the optical density of the red supernatant, if necessary after dilution with water, was measured at 541 mμ using a Beckman type spectrophotometer (Shimazu QB-50 type). By plotting optical density against incubation time a

kinetic curve for hemolysis was obtained, from which $t_{\frac{1}{2}}$ was be obtained graphically. Then, by plotting log $t_{\frac{1}{2}}$ against log V (V is a reciprocal of dilution of C′) a linear relationship (a calibration curve) was obtained. Although a pooled serum was used which was always obtained from several guinea pigs out of 20, the complement activity of guinea pig serum was somewhat different from time to time, and therefore, a calibration curve was prepared concurrently with each set of experiments.

RESULTS

I. Preliminary Experiments

Inactivation of Complement Activity by $NaIO_4$ and Its Protection by Ethylene Glycol of Various Concentrations—(*a*) 0.02 *M* $NaIO_4$ solution was treated with equal volume of ethylene glycol of 0.449 to 0.036 *M* concentrations. The grade of decomposition of $NaIO_4$ by glycol was tested using a hemolytic

TABLE I

Decomposition of $NaIO_4$ by Ethylene Glycol (*i*)

0.4 ml. of 0.02 *M* $NaIO_4$ and 0.4 ml. each of 0.449 to 0.036 *M* of ethylene glycol were mixed and allowed to react for 15 minutes at room temperature. 0.4 ml. of 1 : 3 diluted C′ was then added and mixed. After standing and occasional shaking for 30 minutes at room temperature 0.25 ml. each of the above mixtures was added to 2.0 ml. of EA′ and incubated for 1.5 hours at 37°. After centrifugation the supernatant was diluted to 1 : 10 with distilled water and was read at 541 mμ.

Ethylene glycol	0.4 (0.449 *M*)	0.4 (0.225 *M*)	0.4 (0.180 *M*)	0.4 (0.090 *M*)	0.4 (0.036 *M*)			
0.9% NaCl						0.4	0.8	1.2
0.02 *M* $NaIO_4$	0.4	0.4	0.4	0.4	0.4	0.4	0	0
1 : 3 diluted C′	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0
Hemolysis	###	###	###	###	###	—	###	—
Optical density (1 : 10 dil.)	1.09	1.06	1.01	0.98	0.95	0.01	0.44	0.03

system. As seen in Table I, $NaIO_4$ completely inactivated the complement activity in the absence of glycol protected from the action of $NaIO_4$ greatly along with the concentration of glycol but not completely. (*b*) 1 : 20 diluted C′ was first treated by equal volume of 0.02 *M* $NaIO_4$ for 15 minutes. The excess of $NaIO_4$ was then acted with glycol of varying concentrations (Table II). Not a trace of hemolysis could be seen in all tubes. On the contrary, in a tube where glycol was replaced by 0.9 per cent NaCl solution an intense dark brown hemolysis of the cells occurred. The supernatant after centrifugation gave optical density of 0.3374 when read at 541 mμ with its 1 : 5 diluted solution. Unhemolyzed erythrocytes of all other tubes remained fresh red in color. It seems, therefore, that this hemolysis is not due to the antigen-antibody reaction but due to the unspecific action of $NaIO_4$.

Therefore it is necessary to avoid the presence of an excess of periodate in the hemolytic system. The admittable highest concentration of $NaIO_4$ present in the hemolytic system is evaluated to be 0.0005 *M*, as it is described below.

TABLE II

Decomposition of $NaIO_4$ by Ethylene Glycol (ii)

1 : 20 diluted C′ was treated with equal volume of 0.02 *M* $NaIO_4$ for 15 minutes at room temperature. 0.2 ml. of this mixture was then added to 0.2 ml. each of 0.898 to 0.180 *M* glycol and mixed. After 25 minutes standing, 2.0 ml of EA′ was added to each tube and incubated for 1.5 hours at 37°.

1 : 20 diluted C′ treated with equal volume of 0.02 *M* $NaIO_4$ for 15 minutes.	0.2	0.2	0.2	0.2	0.2
0.9% NaCl					0.2
Ethylene glycol	0.2 (0.898 *M*)	0.2 (0.449 *M*)	0.2 (0.225 *M*)	0.2 (0.180 *M*)	0
Hemolysis	—	—	—	—	卌*

* The color is not fresh red, but dark brown. Its 1 : 5 diluted supernatant gave optical density of 0.34 at 541 mμ. Erythrocytes of all other tubes were fresh red in color.

Decomposition of $NaIO_4$ by Ethylene Glycol with Relating to Timing—The decomposition of $NaIO_4$ by ethylene glycol went with time, *i.e.* the decomposition was 4 times greater at the end of one hour compared with that on the instant (*ca.* 40 seconds) of adding glycol to $NaIO_4$ solution as expressed in terms of optical density (Table omitted).

Effect of $NaIO_4$ on Sensitized Erythrocytes (EA′)—1 ml. of EA′ and 1 ml. each of $NaIO_4$ of various concentrations were mixed and incubated at 37° for 1.5 hours. It was found that 0.01 to 0.001 *M* of $NaIO_4$ in final concentrations gave rise to marked black to dark brown hemolysate, 0.0006 *M* faint dark brown, 0.0005 *M* and lesser concentrations gave no hemolysate after centrifugation (Table omitted).

Effect of $NaIO_4$ on Hemolysin—0.5 ml. of 1 : 100 diluted hemolysin was treated with 0.5 ml. of 0.02 *M* $NaIO_4$ for an instant to 60 minutes. After that time 1.0 ml. of 0.449 *M* glycol was added to decompose the excess of $NaIO_4$ and hemolysis reaction was tested. 1 : 5 diluted supernatants gave optical densities of 1.09 to 1.23, whereas controls gave 1.38 and 1.33 where $NaIO_4$ and glycol in the former, $NaIO_4$ in the latter were replaced by 0.9 per cent NaCl. The results show that $NaIO_4$ acts slightly on hemolysin under the conditions. But the possibility that the remaining $NaIO_4$, as shown in Table I, may act on C′ cannot be excluded.

Effect of Glycol on EA′ and C′—Similar several experiments were performed. The results showed that glycol has no effect on EA′ at all and has a very

slight inhibitory action on C′ in final concentrations of 0.074 to 0.0074 *M*.

From these results it may be concluded that $NaIO_4$ destroys strongly a hemolytic system and a constitutent of which most responsible for this destruction is complement. This destructive action of $NaIO_4$ is greatly reduced by ethylene glycol; Fortunately, the latter has no marked effect on a hemolytic system.

II. Quantitative Aspect of Inactivation of Complement Activity by $NaIO_4$

One volume of fresh C′ was treated with one volume of 0.02 *M* $NaIO_4$ for a moment, 5, 10, 15, 30, 60, 90 and 120 minutes, respectively. The activity of thus treated samples was quantitatively measured with its 1 : 15 diluted equivalent in amount of C′ (Table III). As seen in the Table the activity

Table III

Action of Metaperiodate on Complement Activity

Equal volumes of guinea pig serum (C′) and 0.02 *M* $NaIO_4$ were mixtured. (A)

I. Portions of 0.4 ml. of A were after a given lapse of time poured into a test tube containing 2.6 ml. of 0.449 *M* glycol to stop the action of $NaIO_4$ and mixed. The resulting mixture of all tubes is equivalent to 1 : 15 dilution in concentration of original C′. 1.0 ml. of this mixture was added to 10 ml. of sensitized erythrocytes and its complement activity was measured.

II. The case is the same as above except that a test tube contained 2.6 ml. of a mixture consisted of equal parts of 0.449 *M* glycol and V. b.

III. 0.5 ml. each of A was added to 0.5 ml. of 0.449 *M* glycol. At the end of above procedure 2.0 ml. of EA′ was added to each tube and incubated at 37° for 1.5 hours.

	Lapse of time	Ca 40″		5′	10′	15′	30′	60′	90′	120′		
I	Activity equivalent to dilution of C′	1:29.3		1:58.6		1:70.2	1:71.9	1:77.7	1:81.5			
II		1:33.5		1:61.7	1:63.8	1:69.2	1:72.4	1:74.5		1:81.3		
III	Hemolysis	###	—[1]	###	##	+	—	—		—	##[2]	###[3]
	O. D.	1.05*	0.02	0.95*	0.31*	0.06	0.02	0.01		0.02	0.56*	0.58*

1) 0.9 per cent NaCl in place of glycol.
2) 1 : 5 diluted C′ in place of A.
3) 1 : 5 diluted C′ in place of A and 0.9% NaCl in place of glycol.
* Read with 1 : 5 diluted supernatant.

decreased rapidly to one fourth (1 : 59–62) in the first 5 minutes and then slowly to approach to the lowest activity of one fifth (1 : 75–78) in 60 minutes. When 1 : 5 diluted C′ was used the activity was completely destroyed within 10 minutes as shown later (Columns 2, 3 of Table V). The last experiment in Table III was given for the reference to previous Tables.

III. Which Components of Complement Are Responsible for the Inactivation by $NaIO_4$?

In order to test the adequacy of 5 preparations, R_1-R_4 and heat-inactivated serum, and to investigate the interactions between $NaIO_4$-treated C′ and the 5 preparatsons, all possible combinations between any two preparation were tested. A typical result is given in Table IV. The values on the diagonal line represent potencies of preparations themselves when 0.8 ml. of themselves alone were used. Yeast-treated serum (R_3) and NH_4OH-treated serum (R_4) were not completely inactivated. The others were fully

TABLE IV

Interactions between Four Reagents, Heat-Inactivated and Periodate-Treated Serum

R_1, R_2, R_3, R_4 and heat-inactivated C′ were prepared as described in Methods and all of them were diluted with V. b. to 1 : 16 in concentration of C′. $NaIO_4$-treated C′ was prepared by mixing 1.0 ml. of C′, 1.0 ml. of 0.9 per cent NaCl and 2.0 ml. of 0.02 *M* $NaIO_4$ and allowed to stand for 1.5 hours at room temperature and then diluted to 16 ml. with a mixture of one part of 0.224 *M* glycol and 2 parts of V. b. 2.0 ml. of EA′ was added to a mixture containing 0.4 ml. each of any two preparations and incubated as before.

1 : 16, 0.4 ml. / 1 : 16, 0.4 ml.	End-piece (R_1)	Mid-piece (R_2)	Yeast-treated (R_3)	NH_4OH-treated (R_4)	Heat-inactivated	$NaIO_4$-treated
End-piece (R_1)	− 0.01	### 0.89	+ 0.20	### 0.94	± 0.04	± 0.03
Mid-piece (R_2)		± 0.03	## 0.62	+ 0.05	± 0.04	+ 0.11
Yeast-treated (R_3)			+ 0.05	### 0.71	## 0.35	± 0.03
NH_4OH-treated (R_4)				± 0.04	### 0.91	## 0.34
Heat-inactivated					− 0.01	± 0.03
$NaIO_4$-treated						− 0.02

inactivated. Judging from the results shown by their mutual interactions these five preparations may be qualified and consequently may be used as reagents. $NaIO_4$-treated serum distinctly hemolysed EA′ in combination with NH_4OH-treated serum (R_4). Though the former gave rise to a slight hemolysis also in combination with mid-piece (R_2), it did not act with heat-inactivated C′. From these results it may be concluded that $NaIO_4$ acts on complement as opposed to ammonia, or $NaIO_4$ inactivates C_1' C_2' C_3' completely but does not C_4'.

IV. Quantitative Aspects of the Action of $NaIO_4$ on Complement Components

Relation between Progress of Inactivation and Reaction Time—Using the same 5 preparations described in *Expt. III* the progress of inactivation of complement component by $NaIO_4$ with time of reaction was investigated. Hemolytic systems were all constructed in the same way as in experiment 3 except difference in time during which $NaIO_4$ acted on C′. The results are given in Fig. 1. Here we see that C_1', C_2 and C_3' were completely inactivated by $NaIO_4$ within 30 minutes but C_4' showed a marked resistance

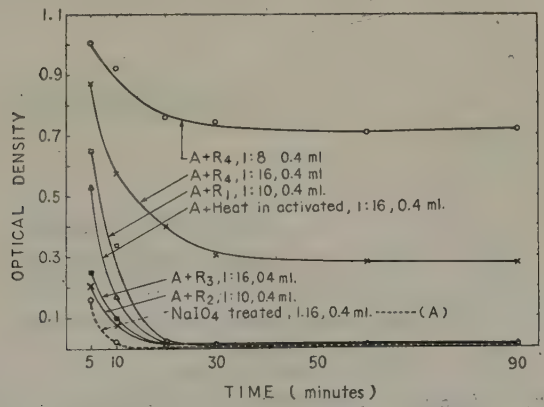

FIG. 1. Effect of $NaIO_4$ on complement components as a function of reaction time.

Five preparations, R_1–R_4 and heat-inactivated C′, were prepared in the same way as in experiment 3 except that their dilutions were slightly varied; R_1 and R_2, 1 : 10; R_3, heat-inactivated C′, 1 : 16; R_4, both 1 : 8 and 1 : 16. A series of $NaIO_4$-treated C′ (A) were also prepared as before except that reaction time during which C′ was acted upon by $NaIO_4$ was varied: 5, 10, 20, 30 and 60 minutes. Abscissa indicates this reaction time. 0.4 ml. each of 1 : 16 diluted A was tested its hemolytic activity in combination with 0.4 ml. each of five preparations.

to NaIO . In this experiment we can see definite cooperation in hemolytic action between R_4 and NaIO -treated C′ (A). A weak cooperation with mid-piece (R_2) suspected in experiment 3 (Table IV) may be ruled out.

Selection of Conditions for Preparing a Preparation of High C_4' Content—It is desirable, in general, that a preparation can be obtained which has no hemolytic activity by itself but has a high content of specified component or components. As previously shown in Table III when one volume of undiluted C′ was acted upon by one volume of 0.02 *M* $NaIO_4$ complement activity decreased but was not completely inactivated even after 2 hours. Therefore, on one hand, one volume of undiluted C′ was treated with more volumes of 0.02 *M* $NaIO_4$ and, on the other hand, diluted C′ was used while the volume of 0.02 *M* $NaIO_4$ was fixed to one volume. Thus the combina-

tions as shown in Fig. 2 were designed.

From these curves it is clear that the best procedure for obtaining a preparation of high C′ content is to treat two volume of 1 : 3 diluted C′ with one volume of 0.02 *M* $NaIO_4$ for 30 to 60 minutes at room temperature in the range of the present experiments. It is too strong in $NaIO_4$ action for the purpose under the conditions of A, B and C and moderate under D in Fig. 2.

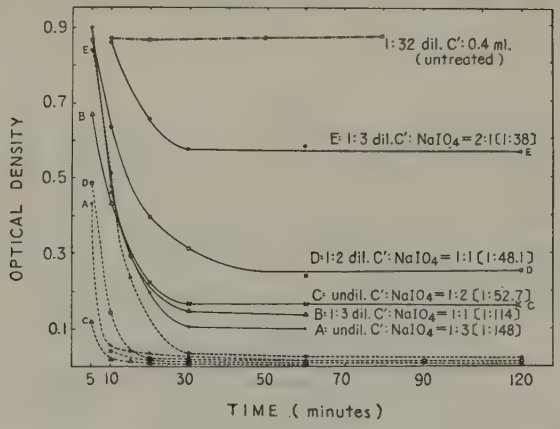

FIG. 2. Selection of conditions for preparing a preparation of high C_4' content.

A, B, C, D and E are the conditions under which 0.02 *M* $NaIO_4$ acted on C′ as indicated in the Fig. For example, E means that two volumes of 1 : 3 diluted C′ was treated by one volume of $NaIO_4$. Under each condition, a series of $NaIO_4$-treated C′ were prepared which differed in reaction time, which is indicated in abscissa. The activities of these preparations were tested in combination with R_4, 0.4 ml. being used, (solid line) and themselves alone, 0.8 ml. being used, without R_4 (broken line). Dilutions of these preparatlons used ranged from 1 : 15 to 1 : 16, E being 1 : 15.75. Dilution of R_4 (ammonia-treated C′) was 1 : 16 and this preparation gave optical density of 0.03 when 0.8 ml. of which was used. In this figure the value of 1 : 32 diluted C′, 0.4 ml. being used, was also given. All the values of optical densities given in Table IV and in Figs. 1 and 2 are those of 1 : 10 diluted supernants.

Further, in order to estimate what these curves represent more concretely, the following experiments were performed. $NaIO_4$ was mixed with C′ in the same way as A, B, C, D and E, and after 35 minutes all were diluted to 1 : 8 in respect to C′ with a mixture composed of equal volumes of 0.448 *M* glycol and V. b. Ammonia-treated C′ (R_4) was also diluted to 1 : 8. The activities were measured quanitatively by adding 0.5 ml. of each preparation and 0.5 ml. of R_4 to 10 ml. of EA′. A calibration curve was also prepared by adding 0.5 ml. of 1 : 16, 1 : 32, 1 : 64, and 1 : 96 diluted C′ to 10 ml. of EA′.

TABLE V

Quantitative Aspects of Interactions between Complement, Metaperiodate, Iodate and Ethylene Glycol

	1	2	3	4	5	6	7	8	9	10	11	12
1:5 dil. C′	1.0	1.0	1.0	1.8	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
0.02 *M* $NaIO_4$		1.0	1.0	1.0	1′	11′	10′	16′	15′		1.0 Ca	1.0 Ca
0.02 *M* $NaIO_3$					1′	1.0	1.0	1.0	1.0		40″	40″
0.449 *M* Glycol		10′ 5′	10′ 5′	15′	-20′- 1.0		7′	11′	15′		4.0′ 1.0	3.0′ 1.0
0.9% NaCl	2.0	1.0	1.0	1.0	1.0	5′ 1.0	1.0	1.0	1.0	4.0		
Above reaction mixture added EA′	1.0 10	1.0 10	1.0 10	1.0 10	1.0 10	1.0 10	1.0 10	1.0 10	1.0 10	1.0 10	1.0 1.0 10	1.0 1.0 10
Hemolysis	###	—	—	###	###	###	###	###	###	###	###	###
Activity corresponding to dilution of C′	1:13.8	unmeasurable		1:18.4	1:37.5	1:14.2	1:17.1	1:16.2	1:16.2	1:25.5	1:37.3	1:31.3

Note: For example, column 4 shows that one ml. of 0.02 *M* $NaIO_4$ was first acted with one ml. of 0.449 *M* glycol for 15 minutes and then one ml. of 1:5 diluted C′ was added and mixed. At the end of one minute one ml. of this mixture was poured into 10 ml. of EA′ and its hemolytic power was quantitatively measured. The result was that strong hemolysis took place and its complement activity proved to be equivalent to 1:18.4 instead of 1:15, diluted C′, and so on.

The activity of A, B, C, D, and E corresponded to that of 1 : 148, 1 : 114, 1 : 52.7, 1 : 48.1 and 1 : 38 diluted C′, respectively. These values were given in brackets in the Fig. For reference, the value of 1 : 42.4 was obtained when 1.0 ml. of D and 0.5 ml. of R_4 were used against the value of 1 : 48.1 just given where 0.5 ml. each of D and R_4 was used. The activities of R_4 and A by themselves, 1.0 ml. each of which being used, were too weak to measure quantitatively.

V. Quantitative Aspects of Interactions between C′, Periodate, Iodate and Glycol

The results are given in Table V. Some of these interactions were already tested in the preliminary experiments. By looking over the Table it may be deduced that $NaIO_4$ strongly destroys C′ activity whereas its decomposition product, $NaIO_3$, does not. Glycol greatly neutralizes $NaIO_4$ and consequently protects C′ from the destructive action of $NaIO_4$, but slightly affects the hemolytic system. It is necessary to note, however, that here the concentrations of $NaIO_4$ and glycol are much higher both in absolute final concentration and in comparison with that of C′ than those used in the main experiments.

The effects of reaction products between metaperiodate and glycol as a whole and of formaldehyde which will be derived from glycol on the hemolytic system were also investigated. That of iodate was just given above. 2.0 ml. of 0.224 *M* glycol was treated with 2.24 ml. of 0.2 *M* (not 0.02 *M*) $NaIO_4$ overnight at room temperature. The activity of 0.5 ml. of 1 : 15 diluted C′ was tested in the presence of 0.5 ml. each of the 1 : 10 diluted reaction mixture mentioned above, 0.008 *M* and 0.0016 *M* formaldehyde solutions, respectively. A calibration curve was prepared concurrently by using 1.0 ml. each of C′ of various concentrations. It was found that the activities were equivalent to that of 1 : 45.2, 1 : 39.8 and 1 : 29.3 diluted C′, instead of 1 : 30, respectively (Table omitted). In the main experiments the amounts of reaction products inevitably brought into 10 ml. of EA′ together with C′ were usually 1 ml. of 0.002 *M* and 0.0013 or lesser according to 1 : 10 and 1 : 15 diluted C′ equivalents used, respectively. When taken account of the latter, the results obtained show that decomposition products of $NaIO_4$ and glycol do not affect appreciably the hemolytic system, and it may be said that the decrease in hemolytic activity of $NaIO_4$-treated C′ may be due to a direct destructive action of $NaIO_4$ against C′.

DISCUSSION

Pillemer and co-workers (*11*) were able to obtain C_1', C_2' and C_4' in pure form, as well-defined proteins, by fractional precipitation of guinea pig serum with ammonium sulfate and dialysis. C_1' was characterized as euglobulin containing 2.7 per cent carbohydrate. C_2' and C_4' were obtained together as an apparently pure mucoprotein containing 10.3 per cent

carbohydrate. Both of these types of complement activity may therefore possibly reside in one and the same molecule. They (*12*) further suggested that C'_2 would be the calcium-carbohydrate-pseudoglobulin molecule, C_1' the carbohydrate, and the complex calcium-pseudoglobulin molecule the carrier of C_4'. Quantitatively, C_1' was contained in guinea pig serum about twice as much as C_2' and C_2' combined (*11*). In addition, it is said that the polysaccharide in blood globulins is composed of units having the composition galactose-mannose-glucosamine (*13*). C_3' was distributed in all the precipitates obtained at the concentrations of 1.39, 1.60 and 2.0 *M* of ammonium sulfate (*11*).

Assuming these carbohydrates to be an essential constituent of the complement components, it was first expected that treatment of serum with metaperiodate would equally results in destruction of C_1', C_2' and C_4', since the reagent (*14*) is well known for its specific ability to oxidize carbohydrate. The experimental results were not in accordance with the expectation.

That amino acids of certain types, such as serine and threonine, are quantitatively oxidized are also well known (*14*). It is not surprising, therefore, that protein may also undergo chemical alteration when it is brought in contact with the reagent. Such facts have actually been proved with gelatin (*15*), crystalline bovine serum albumin (*16*), and biologically active proteins (*17*). Therfore, it may be conceivable that metaperiodate affects the proteins of complement.

That heating of serum at 66° for 30 minutes destroys the activities of all the components of complement (*18*) strongly indicates that the essential constituent of complement is of protein nature, since it is very unlikely that carbohydrate undergoes chemical change through such a mild treatment. From these considerations it may be said that the effect of metaperiodate on complement activity may be largely due to alteration of its protein structure. But the decisive conclusion needs, of course, further experimental evidence. It must also be taken into consideration that there is inherent differences in titer between complement components (*19–22*).

Dilute ammonia (*9*) and amino compounds including hydrazine that are known to have decided reactions with aldehyde (*12*) inactivate C_4'. Metaperiodate fairly leaves C_4' unaffected. In this respect, the effect of metaperiodate on complement is just the reverse of that of ammonia and of the amino compounds.

SUMMARY

1. The effect of metaperiodate on guinea pig complement activity was investigated quantitatively.

2. Metaperiodate destroys strongly a hemolytic system and a factor of which responsible for this destruction is complement. This action of metaperiodate is greatly reduced by ethylene glycol. The latter does not affect a hemolytic system appreciably.

3. The first, second and third complement components (C_1', C_2' and

C_3') are easily destroyed by metaperiodate, but the fourth component (C_4') is fairly resistant to the reagent.

4. The best procedure for preparing a preparation of C_4' is to treat two volumes of 1:3 diluted serum with one volume of 0.02 *M* $NaIO_4$ for 30 to 60 minutes at room temperature and then to neutralize the excess of reagent with ethylene glycol. The activity of this preparation combined with an equal volume of ammonia-treated serum, concentrations of both preparations being 1:8 dilution in terms of original serum, was equivalent to that of 1:38 diluted serum, that is, about one fifth.

5. The mode of action of metaperiodate on complement activity was discussed.

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The Journal of Biochemistry, Vol. 46, No. 3, 1959

BIOCHEMICAL STUDIES ON SULFUR-CONTAINING AMINOACIDS

II. SULFATE FORMATION FROM L-CYSTINE BY MOLDS

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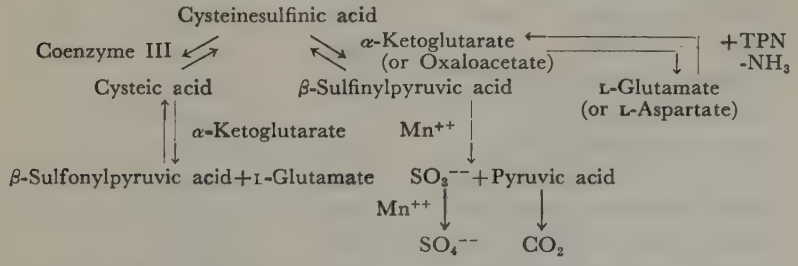
(Received for publication, July 28, 1958)

The authors (*1*) have previously shown the formation of sulfate from L-cystine by *Aspergillus* molds. Other nitrogen sources added to the L-cystine media, in which L-cystine was the sole source of nitrogen, namely amino-acids, ammonium salts, nitrate or urea, diminished the sulfate formation. From cysteate, taurine, dithiodilactate, dithiodiglycol or thioglycolate, which were added respectively as a substitute nitrogen or sulfur source for cystine in the medium, the sulfate formation could not be recognized. Only mercaptopyruvate was the precursor of sulfate. These results led the authors to postulate tentatively the pathways of L-cystine metabolism in some molds as follows:

$$(\text{-}SCH_2CHNH_2COOH)_2 \longrightarrow (\text{-}SCH_2COCOOH)_2$$
$$\longrightarrow HO_2SCH_2COCOOH \longrightarrow SO_4^{--}$$

On the mechanism of the formation of inorganic sulfate by *Microsporum gypseum*, Stahl *et al.* (*2*) reported to pass through cysteinesulfinic acid and then sulfite. Kearney and Singer (*3*) undertook a systematic investigation of the intermediary metabolism of cysteinesulfinic acid in *Proteus vulgaris* with the results obtained as summarized below.

Pathway of Metabolism of Cysteinesulfinic Acid in Proteus vulgaris



Singer and Kearney (*4, 5*) reported an extensive study of the fate of L-cysteinesulfinate in a variety of animal tissues, particularly in mitochondrial preparations, and pointed out a major connection between the sulfate formation and the tricarboxylic acid cycle.

For the research on enzymes formed in the process of sulfate formation by molds, high sulfate productivity of mold was required as enzyme source; this was the reason of this study. Sulfate formation was detected in the L-cystine media of the molds belonging to the following genera: *Scopulariopsis*, *Penicillium*, *Paecilomyces*, *Alternaria*, *Botrytis*, *Cladosporium*, *Monascus*, *Oospora*, *Fusarium* and *Monilia*. However, none of them was able to produce sulfate to the same large degree as *Aspergillus niger*.

EXPERIMENTS

L-*Cystine Medium*—The medium used in this experiment was the Czapeck solution in which L-cystine was substituted for sodium nitrate in accord with the following procedure: 100 ml. Erlenmeyer flasks containing 0.1 g. of L-cystine were supplied with 30 ml. of the sugar-salts solution consisting of 30 g. of sucrose, 1 g. of K_2HPO_4, 0.5 g. of $MgSO_4 \cdot 7H_2O$, 0.5 g. of KCl, 0.01 g. of $FeSO_4$ and one liter of water.

Incubation and Sulfate Determination—After inoculation, each flask was incubated at 37° for 12 days. The mycelial mat was separared from the medium by filtration and washed with distilled water. The filtrate and washings were combined and used for sulfate deter-

TABLE I

Growth and Sulfate Formation of Various Strains of Molds on Media Having L-*Cystine as Sole Nitrogen Source*

Strain	Growth	pH	Sulfate*
Scopulariopsis brevicaulis (SACC.) BAIN.	176 (*mg.*)	4.0–4.2	46 (*mg.*)
Penicillium camemberti THOM	25	3.8–4.0	25
„ *digitatum* SACC.	23	3.6–3.8	19
„ *lilacinum* THOM	180	4.0–4.2	39
„ *notatum* THOM	95	4.0–4.2	35
„ *roqueforti* THOM	224	3.8–4.0	62
„ *yezoensum* HANZAWA	221	3.8–4.0	71
„ *sp.*	229	4.0–4.2	54
Paecilomyces varioti BAIN.	179	4.0–4.2	59
Alternaria tenuis NEES	215	3.6–3.8	63
Botrytis cinerea PERS.	300	3.8–4.0	65
Cladosporium herbarum LINK	280	4.0–4.2	27
Monascus purpureus WENT	77	4.0–4.2	31
Oospora lactis (FRES.) SACC.	21	3.4–3.6	20
„ *suaveolens* (LINDER) LINDAU	34	4.0–4.2	28
Fusarium graminearum SCHWARE	184	4.0–4.2	45
Monilia sitophila (MONT.) SACC.	242	3.8–4.0	44
Aspergillus oryzae (AHLB.) COHN	153	4.0–4.2	58
„ *niger* v. TIEGHEN	142	2.8–3.0	102

* The sulfate was measured as $BaSO_4$; each flask contained 21 mg. of sulfate represented as $BaSO_4$ before inoculation.

mination by gravimetrical method. Growth was measured as dry weight of mycelium. Each figure in Table I represents the average value of three determinations.

RESULTS

Growth and sulfate formation of various strains of molds are presented in Table I. In this incubation, the culture medium of *Cladosporium herbarum* became reddish and the L-cystine was almost entirely consumed. The sulfate formation, however, was very small in amount. Except *Penicillium digitatum* and *Oospora lactis*, every strain tested here formed sulfate from L-cystine but none of them could produce sulfate as well as *Aspergillus niger*.

SUMMARY

Sulfate formation by 19 strains of molds in L-cystine media in which L-cystine was the sole nitrogen source was compared. Except *Penicillium digitatum* and *Oospora lactis*, all of the strains belonging to the genera *Scopulariopsis*, *Penicillium*, *Paecilomyces*, *Alternaria*, *Botrytis*, *Cladosporium*, *Monascus*, *Oospora*, *Fusarium*, *Monilia*, and *Aspergillus* formed sulfate from L-cystine. *Aspergillus niger* showed the highest ability of the sulfate production.

The authors wish to express their thanks to Dr. Y. Sasaki of the Institute of Applied Mycology, Hokkaido University, for his kindly supplying microorganisms used in this study.

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CHEMICAL MODIFICATION OF TAKA-AMYLASE A

II. PHENYLAZOBENZOYLATION OF TAKA-AMYLASE A

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(Received for publication, July 28, 1958)

In a previous paper (*1*) the author reported the chemical modifications of Taka-amylase A (TAA) with fluorodinitrobenzene and dinitrobenzene sulfonate. From these results, it was suggested that ε-amino groups of lysine residues of the protein do not play any essential role for the emergence of amylase activity, but some special phenolic groups of tyrosine residues are more closely connected with the activity.

In the present study, *p*-phenylazobenzoyl (PhAB) chloride was chosen as an acylating reagent to modify TAA, because PhAB compounds exhibit characteristic absorption maxima at 430 and 330 mμ, and the number of the reagent introduced into the protein can be determined with great ease.

Recently, it was confirmed in our laboratory that crystalline TAA is able to hydrolyse α-phenylmaltoside and *p*-nitrophenyl-α-maltoside to form maltose and corresponding phenols when considerably high concentration of the enzyme are used and this α-phenylmaltosidase activity is an intrinsic property of the TAA protein itself (*2*).

In tne present study, the changes of activities of the enzyme by acylation with PhAB chloride were determined using amylose, maltotetraose, α-phenylmaltoside and *p*-nitrophenyl-α-maltoside as substrates, and it was found that the amylase activity is almost lost by introduction of 1 mole of PhAB residue into 1 mole of the amylase protein, while α-phenylmaltosidase activity increases about 2.5 fold, and it was suggested that PhAB residue may be combined with a special group situated in the vicinity of the active center.

EXPERIMENTAL

Taka-amylase A (TAA)—Crystalline TAA was prepared from "Takadiastase Sankyo" according to the method described previously (*3*) and recrystallized three times from aqueous acetone. The average molecular weight of 53,000 (*4*) was employed for TAA in calculating its molar concentration and the number of reagent combined with the protein.

Amylase Activity—Amylase activity was measured at pH 5.3 (acetate buffer), 37° by determining its saccharogenic activity by the method of Fuwa (*5*) using amylose as substrate.

α-Phenylmaltosidase Activity—α-Phenylmaltosidase activity was measured by determining phenol liberated by the enzymatic hydrolysis of α-phenylmaltoside using Folin's phenol

reagent. 1 ml. of enzyme solution and 1 ml. of 0.2 *M* acetate buffer of pH 5.3 were incubated at 37° and the enzymatic action was started by the addition of 0.5 per cent α-phenylmaltoside solution which was already preincubated at 37°. After 30 minutes, 3 ml. of 5 per cent sodium carbonate solution was added in order to stop the enzymatic reaction. The amount of the liberated phenol was determined colorimetrically at 660 mμ after the incubation of the reaction mixture with 1 ml. of the 2-fold-diluted solution of Folin's phenol reagent at 37° for 20 minutes.

When *p*-nitrophenyl-α-maltoside was used as substrate the liberated *p*-nitrophenol could be determined colorimetrically at 400 mμ after the addition of 5 ml. of 5 per cent sodium carbonate solution.

p-Phenylazobenzoyl Chloride—This reagent was prepared by the action of thionyl chloride on *p*-phenylazobenzoic acid (*6*). The latter was synthesized by the condensation of *p*-aminobenzoic acid and nitrosobenzene (*7*).

Phenylazobenzoyl-Amino Acids—1 mM of an amino acid and 2.5 mM of sodium carbonate were dissolved in 10 ml. of water and cooled in an ice bath. To this solution was added 5 ml. of acetone solution containing 1 mM of PhAB chloride. After 4 hours, hydrochloric acid was added to the reaction mixture, and the resultant precipitate was collected, washed with a small amount of water and dried in a vacuum desiccator. To remove phenylazobenzoic acid contaminated in the PhAB-amino acid preparation the precipitate was treated three times with 10 ml. portions of ether and then recrystallized from aqueous acetone.

Melting points of PhAB-amino acids synthesized by this method are as follows:

N-PhAB-glycine	m.p. 221–224°
N-PhAB-alanine	m.p. 211–215°
N-PhAB-serine	m.p. 192°
O-PhAB-*N*-chloroacetyl-tyrosine	m.p. 181°

The absorption spectra of phenylazobenzoic acid and PhAB-amino acids are shown in Fig. 1.

Phenylazobenzoylation of TAA—A mixture of 1 volume of 1 per cent TAA solution (1.9×10^{-4} *M*) and 1 volume of 0.1 *M* buffer was cooled in an ice bath, and then 1 volume of cold acetone was slowly added. To this solution was added PhAB chloride at a molar ratio of 10 or 20 moles of the reagent per mole TAA.

After desired periods of reaction time, 1 ml. portion of the reaction mixture was pipetted out, and diluted to 2,500 ml. with distilled water, and was used for the determination of amylase activity. Another 1 ml. of the reaction mixture was diluted to 150 ml. for the determination of maltosidase activities using α-phenylmaltoside, *p*-nitrophenyl-α-maltoside and maltotetraose as substrates. Still another 10 ml. of the reaction mixture were poured into 3 ml. of 20 per cent trichloroacetic acid. The precipitated PhAB-TAA was centrifuged, washed four times with acetone to remove phenylazobenzoic acid and then three times with 0.1 *N* acetic acid to remove acetone. The PhAB-TAA thus obtained was dissolved in 0.1 *N* sodium hydroxide. The concentration of PhAB residue in the solution was determined spectrophotometrically at 430 mμ or 330 mμ, while the protein concentration was determined by the micro-Kjeldahl method. From these two data, the number of PhAB residue combined with 1 mole of TAA was calculated.

Maltotetraose—A 0.5 per cent amylose solution was partially hydrolyzed at pH 6.0 by the action of a very low concentration of a crystalline bacterial amylase (*8*). As soon as the amylose-iodine color of the solution became no more positive, the reaction was stopped by the addition of trichloroacetic acid to a final concentration of 3 per cent. After filtration, the solution was passed through a Dowex 2 column to remove trichloroacetic

acid. The sugar concentration in the solution thus obtained was 0.35 per cent and the average polymerisation degree was 4.2, which were calculated from the saccharogenic power of the solution before and after acid hydrolysis.

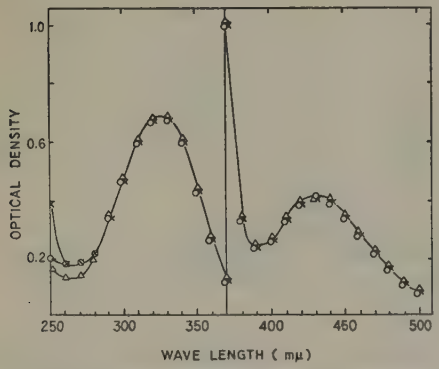

FIG. 1. Absorption spectra of phenylazobenzoic acid and PhAB-amino acids in 0.1 *N* NaOH. —○— PhAB-glycine, alanine, and serine, —×— *O*-PhAB-*N*-chloroacetyl-tyrosine, —△— phenylazobenzoic acid. The concentrations are 3.0×10^{-5} *M* from 250 to 370 mμ, and 3.0×10^{-4} *M* from 370 to 500 mμ.

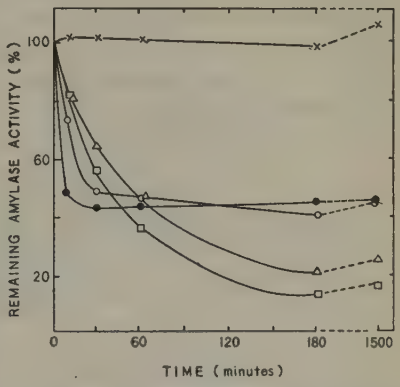

FIG. 2. The change of amylase activity during phenylazobenzoylation at various pH and at molar ratio of 10 moles of the reagent per mole of TAA. —×— control, —●— at pH 9.0, —○— at pH 8.0, —△— at pH 7.0, —□— at pH 6.0.

RESULTS AND DISCUSSION

PhAB chloride was reacted to TAA as described in the experimental part at pH 6.0 (0.1 *M* acetate buffer), 7.0, 8.0 and 9.0 (0.1 *M* veronal buffer) and at molar ratio of 10 moles of the reagent per mole of TAA. The change of enzymatic activity was measured using amylose as substrate. As shown in Fig. 2, the reaction at pH 9.0 took place rapidly and was completed in a relatively short period of time. The PhAB-enzyme thus produced retained 45 per cent of the original amylase activity. The reaction at pH 6.0, on the other hand, proceeded very slowly and resulted in an extensive inactivation of the enzyme. Only 15 per cent of the original activity remained after the treatment.

The absorption spectra in 0.1 *N* sodium hydroxide of the PhAB-TAA obtained by the phenylazobenzoylation at various pH are shown in Fig. 3. The number of PhAB residue introduced into 1 mole of TAA at the completion of reaction was measured photometrically as described in the experimental part and found to be 4.5 moles, 4.1 moles, and 2.6 moles at pH 9.0, 8.0, and 7.0, respectively. At pH 6.0, on the other hand, about 1 mole of PhAB residue was introduced into 1 mole of the enzyme protein.

The relationship between the number of PhAB residue introduced and the remaining amylase activity was determined more precisely during

phenylazobenzoylation at pH 6.0 and 9.0. The results of these experiments are shown in Fig. 4. It is very clear from Fig. 4 that the loss of amylase

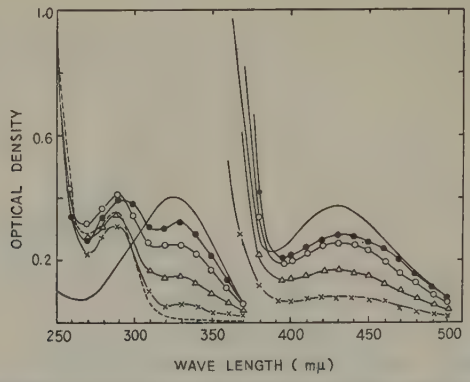

FIG. 3. Absorption spectra in 0.1 *N* NaOH of the PhAB-TAA obtained by the phenylazobenzoylation at various pH and at molar ratio of 10 moles of the reagent per mole of TAA for 25 hours, the original TAA and phenylazobenzoic acid.

—— phenylazobenzoic acid, - - - - intact TAA, —●— PhAB-TAA at pH 9.0, —○— PhAB-TAA at pH 8.0, —△— PhAB-TAA at pH 7.0, —×— PhAB-TAA at pH 6.0.

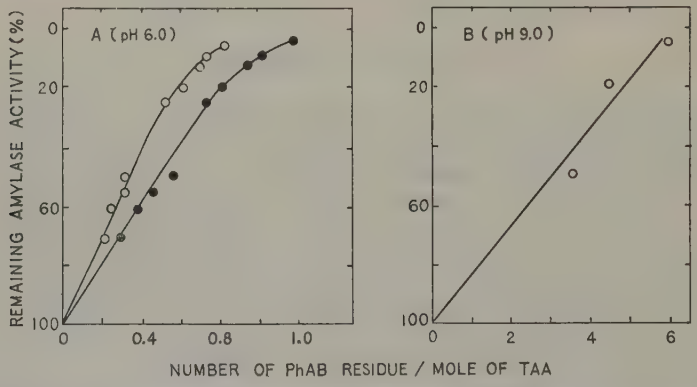

FIG. 4. The relationship between the number of PhAB residue introduced into TAA and the remaining amylase activity during the phenylazobenzoylation at pH 6.0 (A), and pH 9.0 (B) at molar ratio of 10 moles of PhAB chloride per mole of TAA. —○— measured at 430 mμ, —●— measured at 330 mμ.

activity was associated with the introduction of one mole of PhAB residue into one mole of TAA. After the completion of phenylazobenzoylation of TAA at 6.0, calcium acetate was added to a concentration of 0.02 *M* and the PhAB-TAA was precipitated by the addition of ice-cold acetone to 55 per cent concentration. The red-brownish PhAB-TAA was collected by centrifugation, dissolved in 0.02 *M* calcium acetate and crystallized from

aqueous acetone according to the procedure used in the crystallization of intact TAA.

On the other hand, phenylazobenzoic acid and ethyl phenylazobenzoate were reacted to TAA under the same conditions as in the case of PhAB chloride. The enzymatic activity was, however, not affected by this treatment. It is well known that acyl group introduced into phenolic group of tyrosien residue is removed by incubating in slightly alkaline media. During the phenylazo- benzoylation of TAA at pH 6.0, an excess of glycine-NaOH buffer (pH 11) was, therefore, added to the reaction mixture. No restoration of amylase activity, however, could be observed (Fig. 5).

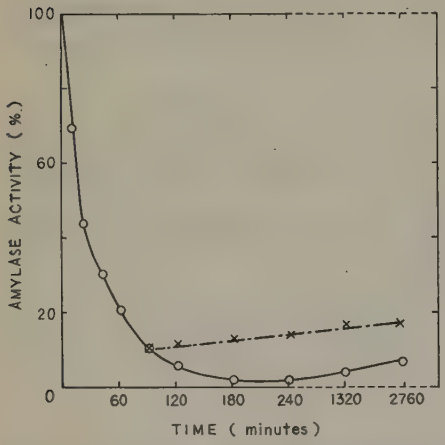

FIG. 5. Observation on the restoration of amylase activity by the addition of glycine-NaOH buffer (pH 11) during the phenylazobenzoylation of TAA at pH 6.0 and at molar ratio of 20 moles of PhAB chloride per mole of TAA.

—○— change of amylase activity during phenylazobenzoylation of TAA, —·—×—·— change of amylase activity after the addition of buffer of pH 11.

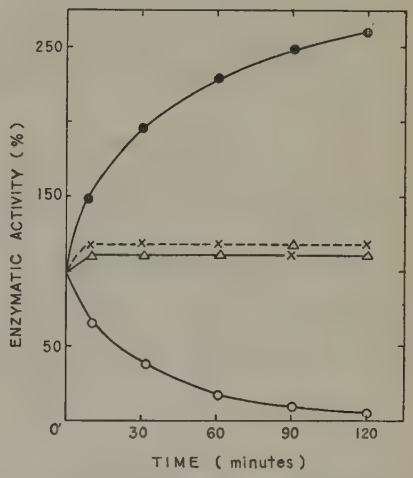

FIG. 6. Changes of the enzymatic activities of TAA toward various substrates during the phenylazobenzoylation at pH 6.0 and at molar ratio of 20 moles per mole of TAA.

—○— toward amylose, —●— toward α-phenylmaltoside, —△— toward *p*-nitrophenyl-α-maltoside, - -×- - toward maltotetraose.

More interesting experimental results were obtained when the changes of the enzymatic activities of TAA toward α-phenyl-maltoside, *p*-nitrophenyl-α-maltoside, and maltotetraose were followed during the phenylazobenzoylation at pH 6.0. As illustrated in Fig. 6, it was found that α-phenylmaltosidase activity of TAA increases about 2.5 fold over the original activity by the phenylazobenzoylation, in spite of the marked decrease in the amylase activity. It is also interesting that the activity of the enzyme to hydrolyse *p*-nitrophenyl-α-maltoside and maltotetraose suffers only a slight change by the phenylazobenzoylation.

These apparently contradictory effects may be understandable if it is assumed that the site of combination of the PhAB residue introduced at pH 6.0 is not the active center itself but a special group situated close to it. Substrates of high molecular weight such as amylose are thus rendered inaccessible to the active site owing to the steric hindrance by the PhAB residue. In the case of α-phenylmaltoside, it seems probable that the interaction between the aromatic moieties of both the substrate and the PhAB residue promotes the formation of the enzyme substrate complex. The active site of TAA for the amylase activity may be different from that for the maltosidase activity. This consideration is excluded because of the difficulty in explaining the fact that the maltosidase activity of TAA increases about 2.5 fold over the original activity. It may further be assumed that such a favorable interaction between the aromatic residues is eliminated in the case of p-nitrophenyl-α-maltoside as substrate due to the influence of the nitro group.

These considerations received a support from experiments in which Michaelis constants of both the intact and PhAB-enzyme toward maltosides were compared. As shown in Fig. 7 and Table I, Michaelis

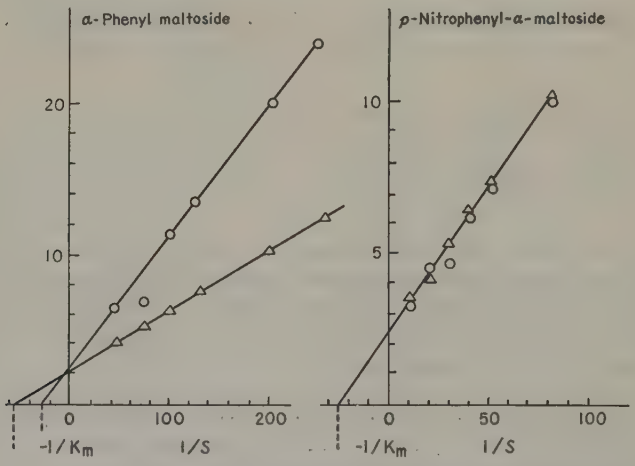

FIG. 7. The relationships between the substrate concentration and the reaction velocities of intact TAA and PhAB-TAA toward α-phenylmaltoside and p-nitrophenyl-α-maltoside respectively. —○— TAA, —△— PhAB-TAA.

constant of intact TAA toward p-nitrophenyl-α-maltoside was found to be 0.04 M, this value exactly identical with that of PhAB-TAA for the same substrate. When α-phenylmaltoside was used as substrate, Michaelis constants of TAA and PhAB-TAA were determined to be 0.04 and 0.02 M, respectively. Based on the above experimental results, it may be inferred that the PhAB residue causes no effect on the rate of decomposition of the enzyme substrate complex to the enzyme and products, but interfers the formation of the enzyme-amylose complex. Experiments to identify the site

of combination of the PhAB residue are now in progress. For this purpose, the PhAB-TAA was hydrolyzed by the action of a protease obtained from *Streptomyces griseus*, the resultant PhAB-peptides were extracted with isoamyl alcohol and separated by paper chromatography using the upper layer of the mixture of equal volume of *n*-butanol and 1 *N* aqueous ammonia as developer. One of the purified PhAB-peptides was hydrolyzed with 6 *N* hydrochloric acid for 20 hours, and its amino acid composition was determined by Stein and Moor's ion exchange chromatography (*9*, *10*). It

Table I

Michaelis Constants of TAA and PhAB-TAA

	α-Phenylmaltoside	*p*-Nitrophenyl-α-maltoside
TAA	0.04 *M*	0.04 *M*
PhAB-TAA	0.02 *M*	0.04 *M*

was found that the peptide contains leucine, isoleucine, valine and lysine. The N-terminal amino acid of the peptide was determined by the Sanger's DNP-method and found to be leucine or isoleucine. From these results, the site of combination of the PhAB residue seems to be a ε-amino group of the special lysine residue situated close to the active site. It seems likely that this particular ε-amino group becomes more reactive to PhAB chloride at pH 6.0 rather than at higher pH.

SUMMARY

1. *p*-Phenylazobenzoyl chloride was reacted with Taka-amylase A at various pH. By the phenylazobenzoylation at pH 6.0, the decrease of amylase activity was associated with the introduction of PhAB-residue. The amylase activity was almost lost by the introduction of about one mole of PhAB residue.

2. The changes of enzymatic activities toward α-phenylmaltoside, *p*-nitrophenyl-α-maltoside and maltotetraose during phenylazobenzoylation was measured at pH 6.0. It was found that α-phenylmaltosidase activity of Taka-amylase A increases about 2.5 fold over the original activity. While the enzymatic activity to hydrolyze *p*-nitrophenyl-α-maltoside and maltotetraose suffers only a slight change by the treatment.

3. It was suggested that the site of combination of the PhAB residue seems to be a ε-amino group of the special lysine residue situated in the vicinity of the active site.

The author wishes to express his gratitude to Prof. S. Akabori for his kind guidance throughout the investigation and to Mr. T. Usami and Mr. Y. Imai for their technical assistance. The author also wishes to thank the Sankyo Co. Ltd. for their kind supply of "Takadiastase Sankyo" and Mr. M. Nomoto for his kind supply of the protease obtained from *Streptomyces griseus*.

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KINETIC ANALYSIS OF THE MYOSIN B-ADENOSINE-TRIPHOSPHATASE SYSTEM*

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Since Engelhardt and Ljubimova (*1*) found the ATPase activity of actomyosin and myosin, its role in muscle contraction has become the subjects of repeated investigations (*2*, *3*). The general properties of this enzyme were elucidated by Banga (*4*), Mommaerts and Seradarian (*5*) and Hasselbach (*6*). These authors showed that Ca^{++} accelerates ATPase under all conditions investigated, while Mg^{++} inhibits ATPase of actomyosin at high KCl concentration and accelerates it at low KCl and also that the pH-activity curve has a characteristic shape showing a maximum and a minimum at pH about 6.5 and 7.5, respectively. More recently, several authors have demonstrated that myosin catalyses the hydrolysis of ITP, GTP, UTP and CTP (*7–11*) in addition to ATP and that it is activated markedly by EDTA (*12–14*), PP (*15*, *16*), AET (*17*), PCMB (*18*) and DNP (*19*, *20*).

Ouellet *et al.* (*21*), Tonomura and Watanabe (*22*, *23*) and Green and Mommaerts (*24*) have already found that ATPase action obeys, in various ionic media, the Michaelis-Menten theory. These kinetic studies have elucidated the mechanism of this enzyme action to some extent. A thorough kinetic study, however, is needed in order to clarify the physico-chemical mechanism of the effect of divalent cations, pH and the particular activators.

In view of these circumstances, the present authors have reinvestigated the kinetic properties of myosin B-ATPase at the steady state** in various ionic media and obtained the following results: (i) Ca^{++} activates ATPase by the chelation of ATP with Ca^{++}, (ii) the effects of divalent cations depend mainly on their ionic radii, (iii) at sufficiently high concentration

* The following abbreviations will be used: ATP adenosine triphosphate, ITP inosine triphosphate, GTP guanosine triphosphate, UTP uridine triphosphate, CTP cytidine triphosphate, NTP nucleotide triphosphate, PCMB *p*-chloromercuribenzoate, AET *S*-β-amino-ethylisothiuronium, DNP 2,4-dinitrophenol, PMA phenylmercuric acetate, EDTA ethylenediamine tetraacetic acid, PP inorganic pyrophosphate, ATPase adenosinetriphosphatase, ITPase inosinetriphosphatase, NTPase nucleotidetriphosphatase, Me^{++} divalent cation and Me^{+}monovalent cation.

** The reaction mechanism of ATPase at the initial stage (*25*, *26*) will be discussed in a subsequent paper (*27*) from this laboratory.

of the substrate, ATPase is activated again by the substrate, (iv) in the presence of high concentrations of the substrate or EDTA, the pH-activity curve lacks the characteristic depression at pH about 7.5, and (v) ITPase bears some resemblance to ATPase in the presence of the particular activator. These results have enabled us to propose a general theory of the reaction mechanism of myosin B-ATPase.

EXPERIMENTALS

Throughout this work the materials and methods used were similar to the standard ones (*22*, *23*) of this laboratory. The 24 hours extracted myosin B (natural actomyosin) was prepared from rabbit skeletal muscle. The protein was stored at $-1\sim0°$, in a concentrated solution using 0.6 *M* KCl of pH 6.4, as a medium, loss of the enzymic activity being scarcely observed during the period of one week. ATP and ITP used were the products of Sigma Co. and EDTA and PCMB were obtained commercially.

The enzyme reaction was started by adding 4 ml. of ATP solution to 16 ml. of the solution, containing myosin B, 0.08 *M* of trisaminomethane maleate or glycine buffer, adequate amounts of KCl and $MeCl_2$ adjusted to the desired pH. The protein concentrations in the reaction mixture ranged from 0.17 to 0.35 mg./ml. At appropriate intervals, aliquots were pipetted into equal volumes of 10 per cent trichloroacetic acid. The precipitated protein was filtered off and the content of inorganic phosphate in the filtrate was analyzed colorimetrically by means of Youngburg-Youngburg's method (*28*). As some variations were found in the maximum velocity and also in the Michaelis constant from preparation to preparation, the authors adopted only the values with the enzyme preparations which showed almost identical values of these constants at least in two sets of the ionic media.

RESULTS

I. ATPase Activity at Low Substrate Concentration

Effect of Divalent Cations—As already demonstrated by Banga (*4*) and Bailey (*29*), myosion A- and myosin B- ATPase are conspicously modified by divalent cations. In the present investigation the effects of various divalent cations (Mg^{++}, Ni^{++}, Co^{++}, Zn^{++}, Fe^{++}, Mn^{++}, Ca^{++}, Sr^{++} and Ba^{++}) were examined at high and low KCl concentrations. Fig. 1 shows the ionic radii (r) of the divalent cations *versur* the ATPase activity (v). At high ionic strength ($\mu=0.6$) the curve v *versus* r showed a sharp maximum at $r=0.95$ A., a deviation of r by 0.35 from 0.95 A. causing almost complete suppression of the ATPase activity. At low ionic strength ($\mu\sim0.15$), on the other hand, the optimal radius remained at 0.95 A., but the slope of the curve $v-r$ became more gentle, so that the activity was reduced to about 50 per cent of the optimum by the deviation of 0.35 A. from 0.95 A. Therefore, it may be concluded that the effects of the divalent cations depend primarily on their ionic radii and that the seemingly strange dependence on ionic strength of Mg^{++} effect is not characteristic to this cation but common to the other divalent cations whose ionic radii are considerably apart from 0.95 A.

As is well known (*21–24*), ATPase action proceeds in good accordance with the Michaelis formula: $v=V_m/(1+K_m/[S])$, where $[S]$ represents the concentration of ATP (Fig. 2). The maximum velocity V_m and the Michaelis constant K_m are estimated in the presence of Ca^{++} or Mg^{++} and at both high (0.6 *M*) and low (0.2 *M*) concentrations of KCl. The results are summarized in Table I and II, where k_2 stands for the value of V_m per ATPase site, that is, V_m (per g. protein) $\times 4.5 \times 10^5$ (*30*). The

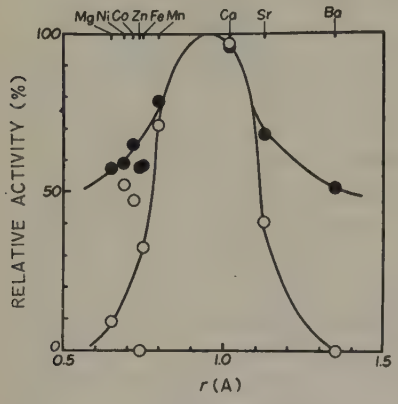

FIG. 1. The relation between the ionic radii (r) of divalent cations and the ATPase activity (v) at high and low ionic strength. The absissa indicates the ionic radius and the ordinate the relative ATPase activity defined v at r=0.95 A. as 100. ●, 0.6 *M* KCl, 5 mM Me^{++}; ○, 0.004 *M* KCl, 1 mM Me^{++}. 1 mM ATP, 22°, pH 8.2 (tris-maleate buffer, 0.008 *M*).

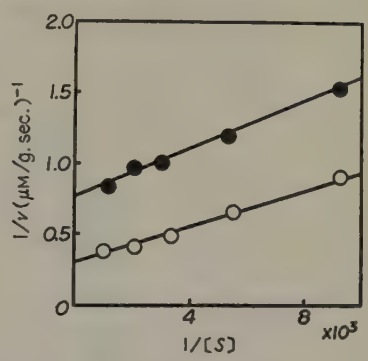

FIG. 2. The dependence of the rate of ATPase (v) on ATP concentration ($[S]$) in the presence (○) and the absence (●) of 7 mM Ca^{++}. 0.6 *M* KCl, 25°, pH 6.7.

TABLE I

Effects of Divalent Cations on V_m and K_m at High Ionic Strength

0.6 *M* KCl, pH 6.7, 25°.

	V_m (μM. g^{-1}. sec^{-1})	k_2 (sec^{-1})		K_m ($\times 10^{-4}$ *M*)	
		Observed	Calcd.	Observed	Calcd.
No modifier	1.3	0.59	(0.59)	1.1	(1.1)
Mg^{++} 1.0 mM	1.3	0.59		1.0	
Mg^{++} 7.0 mM	0.8	0.36		1.0	
Ca^{++} 1.0 mM	2.0	0.86	0.69	1.3	1.26
Ca^{++} 2.8 mM	2.2	0.95	0.97	1.5	1.94
Ca^{++} 7.0 mM	3.2	1.5	1.6	2.1	2.1
(Ca^{++} ∞)		1.7	(1.7)	2.4	(2.4)

TABLE II

Effects of Divalent Cations on V_m and K_m at Low Ionic Strength

	V_m (μM. g^{-1}. sec^{-1})	k_2 (sec^{-1})	K_m ($\times 10^{-4} M$)
No modifier	2.0	0.9	2.5
Mg^{++} 1.0 mM	1.9	0.86	2.5
Mg^{++} 7.0 mM	1.4	0.63	2.3
Ca^{++} 1.0 mM	4·0	1·8	3.0
Ca^{++} 7.0 mM	6.7	3.0	3.4

TABLE III

Effects of KCl Concentration on k_2 and K_m

7 mM Ca^{++}, pH 6.6, 25°.

KCl (M)	k_2 (sec^{-1})	K_m ($\times 10^{-4} M$)
0.6	1.5	2.1
0.4	2.1	2.9
0.2	3.1	3.4
0.1	3.9	3.9

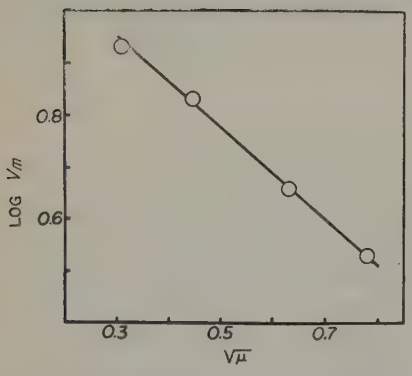

FIG. 3. Plot of log V_m *versus* square root of the ionic strength. 1 mM Ca^{++}, 25°, pH 6.7.

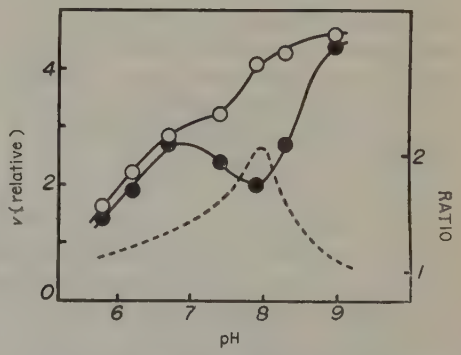

FIG. 4. The pH-activity curve of ATPase. ○, 16 mM ATP; ●, 1 mM ATP. The dotted line represents the ratio of the rates at these two concentrations. 0.6 M KCl, no modifier, 23°.

values of V_m are found to be smaller than those reported by Ouellet *et al.* (*21*) and by Green and Mommaerts (*24*) but they are almost identical with that of Bowen and Gershfeld (*31*). Table III illustrates the dependence of V_m and K_m on the ionic strength. As the ionic strength decreased, both V_m and K_m increased. The plot of log V_m *versus* square

root of the ionic strength gave a straight line (Fig. 3), confirming the previous results of Laidler and Beardell (*32*).

pH Dependence—In the presence of 1 mM ATP the pH-activity curve showed a maximum at pH 6.8 and a minimum at pH 7.8 (Fig. 4), as was first reported by Banga (*4*). These extremes were also observed even when the concentration of the substrate was decreased to a value much smaller than K_m, *i.e.* to 10 μM.

In Fig. 5 are shown the variations of K_m under two ionic conditions: one was in 0.6 *M* KCl and 7 mM Ca^{++} and the other in 0.2 *M* KCl and 1 mM Ca^{++}. Contrary to v, K_m was scarcely changed in the range of pH between 6.7 and 8.0. However, at pH lower than 6.7, K_m increased with the decrease of pH.

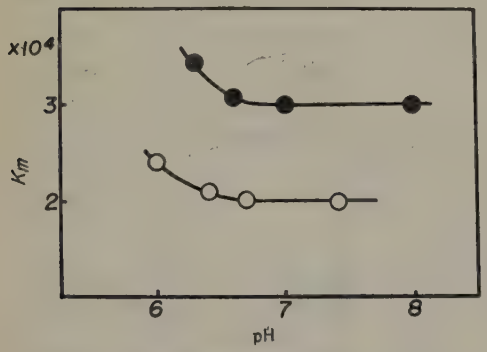

FIG. 5. The pH dependence of the Michaelis constant at 0.6 *M* KCl and 7 mM Ca^{++} (○) and at 0.2 *M* KCl and 1 mM Ca^{++} (●), 25°.

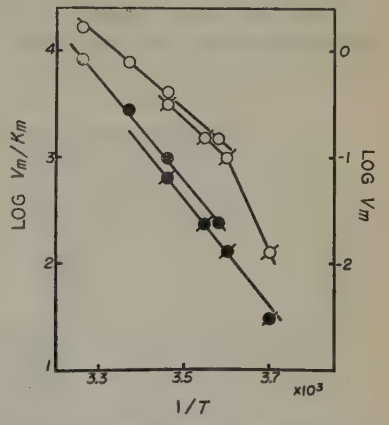

FIG. 6. Dependence of log V_m (open circles) and log (V_m/K_m) (closed circles) on $1/T$. 0.6 *M* KCl, 7 mM Ca^{++}, pH 6.7 (at 15°). ○ and ● represent $\log \frac{1}{2} V_m$ and $\log (V_m/K_m)$ respectively, in the presence of 20 per cent glycerol.

Temperature Dependence—Temperature dependence of ATPase in 0.6 *M* KCl and 7 mM Ca^{++} is illustrated in Fig. 6 by plotting log V_m as well as log (V_m/K_m) against reciprocals of absolute temperature. The experiments below 0° were conducted in reaction mixtures containing 20 per cent glycerol. The presence of glycerol suppressed V_m and K_m, but gave no effect on the temperature coefficient within the range of temperature investigated. As clearly shown in Fig. 6, the temperature coefficient of V_m below 0° was somewhat larger than that above 0°. Similar phenomenon has already been recognized in the cases of urease (*33*), phosphatase and peroxidase (*34*).

II. ATPase Activity in the Presence of EDTA, PCMB or High Concentrations of Substrate

Inhibition and Activation by Substrate—Heinz and Holton (*35*) and

Hasselbach (*6*) have found that the high concentration of ATP suppressed ATPase activity. In Fig. 7 is shown the relationship between v and $[S]$ in the range of $[S]$ from 0.1 to 4 mM at pH 8.2 and in 0.6 M KCl solution. The solid line in this figure has been calculated from the following equation;

$$v=\frac{V_m}{\left(1+\frac{K_m}{[S]}\right)\left\{1+\left(\frac{[S]}{K_m'}\right)^2\right\}} \qquad (1)$$

where $K_m=10^{-3.5}$ and $K_m'=10^{-2.6}$. The agreement of the calculated curve with the experimental results is satisfactory in this range of $[S]$.

One of the most distinctive features of ATPase observed by us was its activation by the substrate; that is, when $[S]$ became higher than 6 mM, ATPase was again activated by the substrate. This substrate activation was more remarkable in the absence than in the presence of divalent cation, as shown in Fig. 8.

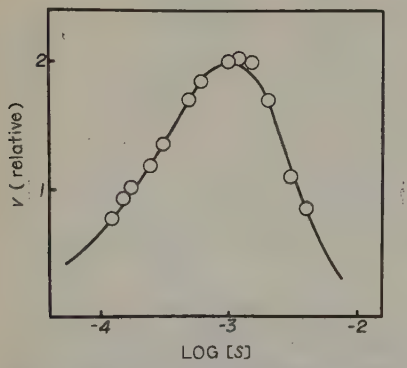

FIG. 7. Substrate inhibition of ATPase. 0.6 M KCl, 25°, pH 8.2. The solid line is the theoretical curve (see text).

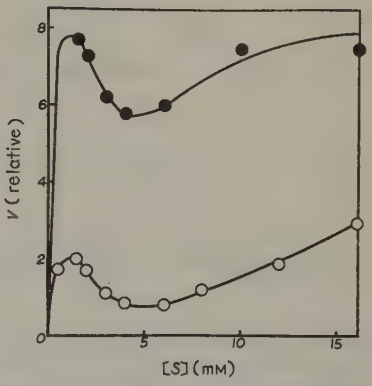

FIG. 8. Activation of ATPase by the substrate in the absence (○) and presence (●) of about 4 mM of free Ca^{++}. 0.6 M KCl, pH 8.2, 25°.

Figs. 9 and 10 illustrate the Lineweaver-Burk plots (*36*) of ATPase action in the presence of 10 mM EDTA and of 40 μM PCMB (per g. protein) plus 4 mM of free Ca^{++}, respectively. In the presence of these activators, neither inhibition nor the activation by the substrate was observed and the plots showed good straight lines. The K_m was increased remarkably by the activators, *e.g.*, the K_m in the presence of 10 mM EDTA and of 40 μM PCMB (per g. protein) plus 4 mM of free Ca^{++} were both 3.3 mM, that is, much higher than the control value, 0.1–0.2 mM.

Effect of Divalent Cation—The dependence of the ATPase activity on the ionic radii of divalent cations was studied in the presence of PCMB. As shown in Fig. 11, in this case, too, the $v-r$ curve assumed a bell-shaped form, but it was somewhat less steep than in the absence of PCMB, though the optimal ionic radius remains at 0.95 A.

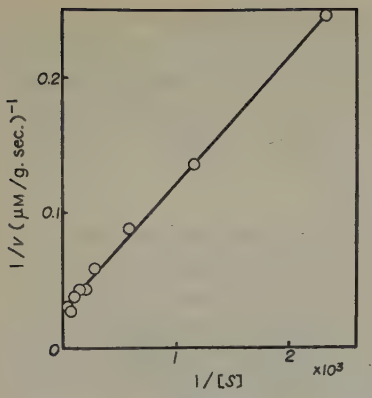

FIG. 9. The Lineweaver-Burk plot of ATPase in the presence of 10 mM EDTA. 0.6 *M* KCl, pH 8.2, 24°.

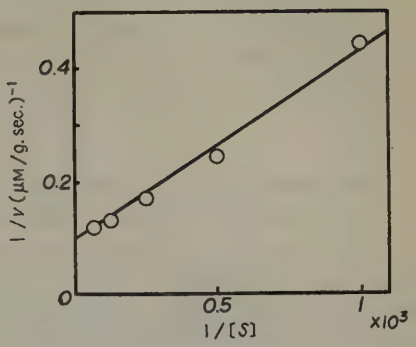

FIG. 10. The Lineweaver-Burk plot of ATPase in the presence of PCMB. 0.6 *M* KCl, 4 mM of free Ca^{++}, 40 μM PCMB per g. protein. pH 8.2, 24°.

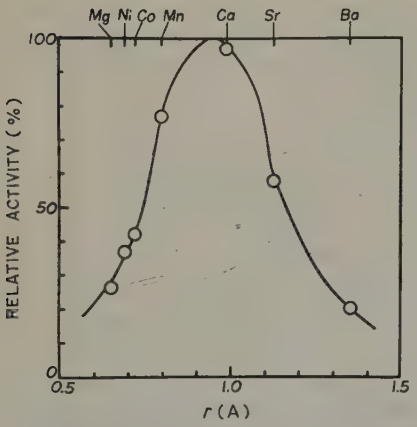

FIG. 11. The relation between the ATPase activity and the ionic radii of divalent cations in the presence of PCMB. 0.6 *M* KCl, 40 μM PCMB per g. protein, 5 mM Me^{++}, 1 mM ATP, pH 8.2, 23°.

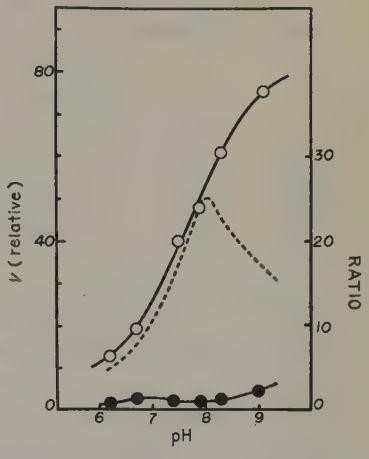

FIG. 12. The pH-variation of ATPase in the presence (○) and absence (●) of 10 mM EDTA. 1 mM ATP, 0.6 *M* KCl, 24°. The dotted line indicates the ratio of the activity in the presence to the one in the absence of EDTA.

pH Dependence—Bowen and Kerwin (*13*) and Maruyama (*37*) observed that in the presence of EDTA the pH-variation was different from the one in the absence of EDTA, *i.e.*, the activity increased in a conventional way to a saturated value, as was confirmed here (Fig. 12). When the ratio of the ATPase activity in the presence of EDTA to that in the absence of EDTA was plotted against pH, a bell-shaped curve with a maximum at pH about 8.0 was obtained.

The pH dependence of the ATPase activity in the presence of a high concentration (16 mM) of ATP was somewhat similar to the one in the presence of EDTA, showing no extreme at neutral pH, as shown in Fig. 4. The ratio of the ATPase activity in the presence of 16 mM to that of 1 mM ATP gave a bell-shaped curve, having its maximum at pH near 8.0.

III. ITPase Activity

The relation between the myosin B-ITPase activity and the ionic radii of divalent cations is diagrammed in Fig. 13. In a way similar to the case of ATPase activity, it gave a bell-shaped curve but its slope was rather gentle, that is, neither Ba^{++} nor Mg^{++} supressed the ITPase action. In this case, the optimal radius was found to be 0.9 A., which was slightly smaller than the value 0.95 A found in the case of ATPase activity.

As previously observed by Spicer and Bowen (*38*), the pH-activity curve of ITPase seemes to have no extreme at neutral pH (Fig. 14). It

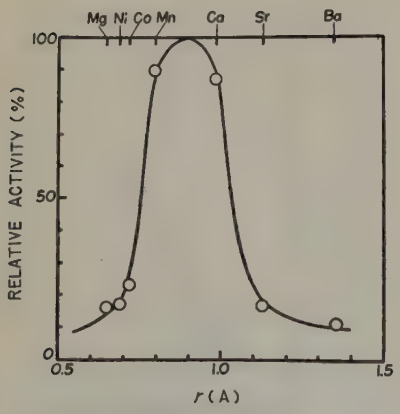

FIG. 13. The relation between the ionic radii (r) of divalent cations and the ITPase activity (v). The absissa indicates the ionic radius and the ordinate the relative ITPase activity defined v at $r=0.9$ A. as 100. 0.6 *M* KCl, 5 mM Me^{++}, 1 mM ITP, pH 8.3, 23°.

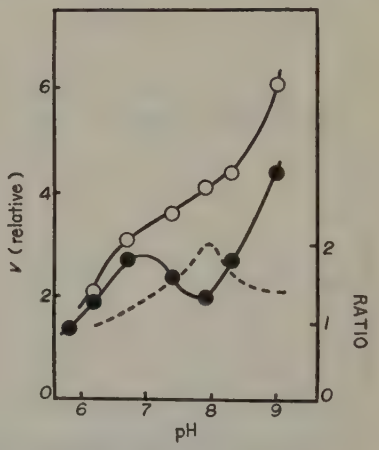

FIG. 14. The pH-activity curves of ITPase (○) and ATPase (●). 1 mM ITP or ATP, 0.6 *M* KCl, 23°. The dotted line indicates the ratio of the ITPase to the ATPase activity.

may be noteworthy that the pH-dependence of ITPase activity was similar to that of ATPase activity activated by high concentrations of ATP. Furthermore, PCMB activated ITPase only slightly, in the presence of 0.6 *M* KCl and 5 mM Mn^{++}, PCMB (59 μM/g. protein) caused only about 25 per cent activation of ITPase, while it accelerated the ATPase about 80 per cent.

DISCUSSION

As mentioned above, ATPase action proceeds following the Michaelis

kinetics under various ionic media. Here attempts are made to analyze the effect of Ca^{++} on ATPase, which was measured rather thoroughly in the present experiments. Taking into account the chelation of ATP with Ca^{++} reported by Smith and Alberty (*39*), the mechanism of the effect of Ca^{++} may be pictured as follows:

$$S + Ca^{++} \underset{K_{ca}}{\rightleftarrows} SCa \tag{2}$$

$$M + SCa \rightleftarrows MCaS \xrightarrow{k_2} M + P \tag{3}$$

$$M + S \rightleftarrows MS \xrightarrow{k_2'} M + P \tag{4}$$

In these equations, M stands for the functional unit of myosin B (unit weight 4.5×10^5 (*30*)), P for products. K and k's are the dissociation constant and the velocity constant of each step, respectively. If the Michaelis constants of steps (3) and (4) are denoted by K_m and K_m', respectively, the steady-state kinetics leads to the usual Michaelis equation as follows:

$$v = \frac{k_2^{\circ}}{1 + \frac{K_m^{\circ}}{S}} \tag{5}$$

where

$$k_2^{\circ} = \left\{ k_2 + k_2' \frac{K_m\left(1 + \frac{K_{ca}}{[Ca]}\right)}{K_m'\left(1 + \frac{[Ca]}{K_{ca}}\right)} \right\} \Big/ \left\{ 1 + \frac{K_m\left(1 + \frac{K_{ca}}{[Ca]}\right)}{K_m'\left(1 + \frac{[Ca]}{K_{ca}}\right)} \right\} \tag{6}$$

and

$$K_m^{\circ} = \left\{ K_m\left(1 + \frac{K_{ca}}{[Ca]}\right) \right\} \Big/ \left\{ 1 + \frac{K_m\left(1 + \frac{K_{ca}}{[Ca]}\right)}{K_m'\left(1 + \frac{[Ca]}{K_{ca}}\right)} \right\} \tag{7}$$

Among these constants, K_m' and k_2' can be determined directly from the measurements in the absence of Ca^{++}, and K_m and k_2 are given as the saturation values by extrapolation to the high concentration of Ca^{++}, neglecting the over-optimal inhibition caused by Ca^{++} (see below), Applying these values and K_{ca}, which is assumed to be 3×10^{-3}, to equations (6) and (7), the Michaelis constant K_m° and the maximum velocity k_2° were calculated and found to agree satisfactorily with the observed ones in the range of Ca^{++} investigated (see Table I). According to Smith and Alberty (*39*), at 0.6 *M* KCl and pH 6.7

$$[\Sigma ATP^{3-}] \ll [\Sigma ATP^{4-}] \fallingdotseq [ATP^{4-}Ca^{++}] + [ATP^{4-}K^{+}] \text{ and}$$
$$[Ca^{++}][ATP^{4-}K^{+}]/[ATP^{4-}Ca^{++}] \fallingdotseq 3.5 \times 10^{-3.0},$$

which is almost equal to the dissociation constant of step (1) deduced here kinetically. This fact may be a good evidence for the proposed mechanism.

Provided that the pyrophosphate group of ATP associates to the ATPase site by mediation of Me^{++}* (*cf.* ref. (*40*)), it may be expected that at high

* In the absence of divalent cations, K^+ may mediate the binding of ATP to the protein.

This provisition may be supported by the following observations made in our laboratory; diphenyl hydrogen pyrophosphate (kindly supplied from Prof. Y. Mizuno of this Univ.) has no effect on ATPase and the physico-chemical properties of myosin B.

concentrations of Me^{++}, ATPase is inhibited being due to the combination of Me^{++} both with the substrate and the active site. As described in a previous paper (*23*), ATPase is inhibited by extremely high concentrations of Ca^{++} and the relation between the degree of the inhibition and the Ca^{++} concentration obeys the dissociation curve of the first order. It was also observed that the $[Mg^{++}]$-activity curve of ATPase could be regarded as a sum of two dissociation curves: the dissociation constant of the first step, where Mg^{++} caused inhibition at high ionic strength and activation at low ionic strength, was much smaller than the dissociation constant of the complex $ATP^{4-}Mg^{++}$, while the dissociation constant of the second inhibiting step was almost equal to that of the complex. Therefore, it may be concluded that, contrary to the case of Ca^{++}, Mg^{++} modifies ATPase by its combination to the enzyme in the first step and it inhibits ATPase by its complex-formation with the substrate in the second one.

The constant value of K_m in the range of pH between 6.7 and 8.0 might be explained by assuming that the concentration of the 'true' substrate in this range of pH is independent of pH and the concentration of active site of the enzyme changes by combination of H^+ in such a way that the reaction rate of step (3) and/or (4) becomes zero. Below 6.7, however, K_m apparently increases with the decrease of pH. Under our experimental conditions the values of $[\Sigma ATP^{4-}]/([\Sigma ATP^{4-}]+[\Sigma ATP^{3-}])$ are as follows (*39*): in 0.6 *M* KCl and 7 mM Ca^{++} solution they are 0.95 and 0.6 at pH 7.0 and 6.0, respectively, and in 0.2 *M* KCl and 1 mM Ca^{++} they are 0.85 and 0.3 at pH 7.0 and 6.0, respectively. Therefore, the dependence of K_m on pH can be interpreted by assuming that ATP^{4-} is the 'true' substrate.

As shown in Figs. 8 and 9, ATPase is depressed by the substrate at concentrations higher than 1 mM, while it is again activated when the concentration of ATP becomes higher than 6 mM. This substrate activation seems to be due to the same mechanism as the EDTA activation; *i.e.* the chelation with the 'intrinsic' Mg^{++} (*14*), since the substrate activation is inhibited by the addition of Ca^{++} and particularly it is not observed in the presence of EDTA.

The effects of the chelating compounds, EDTA, PP, ATP, AET and the sulfhydryl reagents, PCMB, upon ATPase share the following common characteristics: (i) the increase of V_m caused by them is accompanied by the increase of K_m (Figs. 9 and 10, ref. (*41*)), (ii) in their presence, the pH-activity curve lacks the depression at neutral pH, and the ratio of the rate in the presence to the one in the absence of these activators *versus* pH gives a bell-shaped curve and its optimum appears at pH about 8.0 (Figs. 4 and 12, ref. (*13*, *16*, *17*)). To explain these facts and the characteristics of myosin B-NTPase, which will be discussed later, the following reaction scheme may be proposed as the molecular mechanism of NTPase;

$$\begin{array}{ccccc} & & k_1 & & k_2 \\ M+S & \underset{k_{-1}}{\rightleftarrows} & M_1S & \longrightarrow & M+P \\ & & \downarrow\uparrow K & & \\ & & M_2S & & \end{array} \qquad (8)$$

where M stands for the functional unit of the enzyme containing two sort of sites 1 and 2: site 1 is the locus where the pyrophosphate group of ATP combines, as stated above, by the mediation of Me^{++} or Me^{+} and site 2 is another locus of binding the pyrophosphate group; the nature of the latter site will be discussed later. If we assume that the equilibrium between M_1S and M_2S is attained satisfactorily fast, the reaction velocity can be given by

$$v=\frac{V_m}{1+\frac{K_m}{[S]}} \tag{9}$$

$$V_m=k_2\varepsilon/(1+K) \tag{10}$$

$$K_m=(k_2+k_{-1})/k_1(1+K) \tag{11}$$

where K is the equilibrium constant of the step $M_1S \rightleftarrows M_2S$ and ε is the total concentration of site 1. Thus, if the equilibrium of the step $MS \rightleftarrows M_2S$ is maintained at right hand side, and a substance combines to or near site 2, hindering the formation of M_2S but more or less unaffecting the other steps, it is immediately apparent that V_m* and K_m** is increased by the addition of such a substance, because in the presence of the activator

$$V_m'=k_2'\varepsilon \tag{12}$$

$$K_m'=(k_2'+k_{-1}')/k_1' \tag{13}$$

where the dots denote the corresponding values in the presence of an activator.

If we adopt a plausible assumption that the pH-dependence of k_2 is unaffected by the addition of the activator, the pH-dependence of k_2 is represented by the one of V_m'***. The lines in Fig. 15 are those calculated from the assumptions that k_2 is unaffected by the activator, that $k_2=k_2^\circ/(1+[H^+]/10^{-6.7})$ and that $K=9/(1+[H^+]/10^{-8.0}+10^{-8.6}/[H^+])$. In general, they are in good accordance with the pH-activity curves reported in this paper and by Morales *et al.* (*17*) and also by Uchida (*16*)****. Thus, the complicated curves of pH-activity can be pictured as the ratio of the

* It is to be noted here that when $k_2 \gg k_2'$, ATPase would be inhibited by a substance which combines to, or attaches near, site 2.

** K_m may be increased also by the repulsion between the substrate and the activator.

*** Another possible explanation of the pH-activity curve in the presence of an activator would be given by assuming that only the dissociated form of the activator is effective. However, this mechanism is unlikely, since in the presence of various activators such as EDTA, ATP, PP and AET, ATPase gives almost the same pH-activity curves. Furthermore, if this mechanism were correct, ATPase in the presence of 10 mM EDTA would not depend on pH, since EDTA activation in the presence of a moderate concentration of ATP is almost independent of its concentration in the range from 0.1 to 10 mM (*13*).

**** In a previous paper (*22*) from this laboratory, the pH-dependences of ATPase was explained by the dissociation of ATP bound to the protein. If this were true, the pH-activity curve would be shifted to more acidic side by the addition of Ca^{++}, but such a change has not been observed. Consequently the previous mechanism proposed must be abandoned.

curves of the pH-dependences of the two steps, both being the ones most frquently observed in enzyme kinetics.

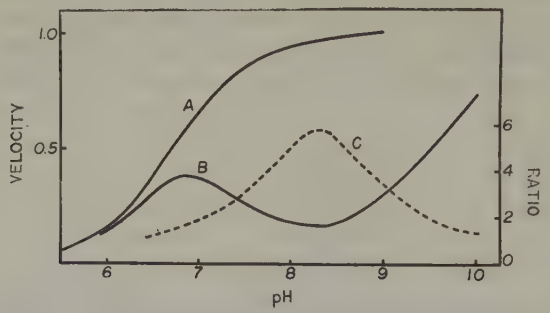

FIG. 15. The theoretical curves of the pH-dependence of myosin B. Curve A, in the presence of an activator; Curve B, in the control condition; Curve C, the ratio of A to B.

Recently, Melchior (*42*) suggested that the molecular configuration of ATP is changed markedly by the ionic radius of metal ion which is chelated by ATP. The bell-shaped curve obtained by the plot of v *versus* r, having its maximum at 0.95 A, may indicate the close relation between the rate of the degradation of the enzyme-substrate complex M_1S and the configuration of ATP, since the $v-r$ curve is changed only slightly by the addition of PCMB, which affects the step, $M_1S \rightleftarrows M_2S$. The above mentioned fact that, when the ionic strength becomes low, the slope of the $v-r$ curve tends to be more gentle, may indicate that the molecular conformation near the active site gets loose by the interaction between myosin and actin.

Blum (*8*) and one of the present authors (*41*, *43*) have proposed the following reaction mechanism for the interaction of myosin B with ATP:

$$\begin{array}{ccccc} M+S & \rightleftarrows & MS & \longrightarrow & M^*S \longrightarrow M^*+P \\ & & \downarrow & & \\ & & M+P & & \end{array} \qquad (14)$$

where M* represents the functional unit of myosin B whose size and shape (*2*, *3*) are changed by ATP. Provided that M_1S in equation (8) corresponds to MS in the above equation and M_2S in equation (8) is a intermediate complex of the step $MS \rightarrow M^*S$, the two mechanisms, equations (8) and (14), turn out to be equivalent. In other words, if one assumes that the binding of ATP to site 2 is a step necessary to the change of the size and shape of myosin B, one can explain the various observations based on the relation between ATPase activity and the physical change of myosin B as discussed by Blum (*8*) and Tonomura (*22*, *41*, *43*).

Accordingly, if the rates of change in the size and shape of M_2NTP are independent of the nucleotide structure, it is reasonable to suppose that the order of the apparent dissociation constants of NTP's for the physical change must be equal to the one of K_m of NTPase (see equation (11)). In fact Blum (*8*) and Kielley *et al.* (*9*) have reported that the order of K_m of

myosin A- and myosin B-NTPase is ITP>GTP>UTP>ATP, CTP. On the other hand, Blum (*8*) and Hasselbach (*11*) have reported the order of the dissociation constants of the physical change to be ITP>UTP>ATP, CTP. It may also be concluded from equations (10) and (11) that the smaller the value of K_m of a NTPase, the more conspicously the NTPase is activated by a substance that interferes with site 2. Actually, EDTA (*9*, *11*) DNP and PMA (*44*) activate ATPase and CTPase strongly, and UTPase slightly but not ITPase. As stated above, PCMB activates ATPase but does ITPase only slightly. The observation (*9*) that EDTA increases K_m of ATPase and CTPase but not K_m of ITPase is also consistent with the present mechanism.

One of the logical deductions from the mechanism proposed here is that the pH-activity curve of a NTPase sensitive to the particular activators shows a maximum and a minimum at neutral pH and the pH-activity curve of a NTPase which is insensitive to the activators lacks these extremes. This situation is also confirmed with ATPase and ITPase*, thus adding more support to the mechanism proposed. In conclusion the reaction scheme proposed in this article offers satisfactory explanations not only for the various observations reported here but also for the various kinds of findings thus far made on NTPase of myosin B.

SUMMARY

1. The effects of divalent cations on myosin B-NTPase activity depend primarily on their ionic radii (r). The $v-r$ curve assumes a bell-shaped form, having its maximum at 0.95 and 0.9 A for ATPase and ITPase, respectively. At low ionic strength the $v-r$ curve is less steep than the one at high ionic strength.

2. Kinetic analysis of the effect of Ca^{++} leads us to the conclusion that Ca^{++} activates ATPase by its complex-formation with ATP.

3. From the pH dependence of K_m, it is concluded that ATP^{4-} is the true substrate of ATPase.

4. The temperature coefficients of V_m and K_m of ATPase are measured at a wide range of temperature. The log $V_m - 1/T$ relation is found to bend at about 0°.

5. When the concentration of ATP becomes higher than 1 mM, ATPase is inhibited, while, at still higher concentrations of ATP (>6 mM), activation of ATPase takes place.

6. EDTA and PCMB increases V_m and K_m of ATPase, and simultaneously they eliminate the substrate-inhibition and -activation.

7. The pH-activity curves of ATPase in the presence of EDTA and of high concentrations of ATP does not show the depression at neutral pH, which is one of the characteristics of the myosin-ATPase under ordinary conditions.

* It should be noted that the rapid falling-off of ITPase at acidic side may be partly due to the formation of ITP^{3-}.

8. ITPase is scarecely activated by PCMB and its pH-activity curve lacks the depression at neutral pH.

9. Based on these results, a mechanism is proposed for the interaction between NTP and myosin B. This mechanism gives satisfactory explanations not only for the observations reported here but also for various other phenomena observed thus far with the myosin B-NTP system.

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CRYSTALLINE CYTOCHROME C

V. CRYSTALLIZATION OF CYTOCHROME C FROM WHEAT GERM*

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Details of the methods used for crystallization of cytochrome c from many animal muscles and beef kidney have been described in the preceding four reports (*1–4*). The method employed for crystallization from bakers' yeast was also reported (*5*). Yakushiji (*6*) and Goddard (*7*) purified cytochrome c from a laver (*Porphyra tenera*) and wheat germ respectively. Purification from wheat germ was not easy due to the low content of cytochrome c, to difficulty in its extraction and also to the presence of many impurities having similar adsorption characteristic to cytochrome c. However, by the improved extraction method and careful treatment with the resin, the pigment was crystallized. This was already reported as a short communication (*8*). In this report the details of the method and its results will be reported.

MATERIALS AND METHODS

Wheat Germ—The material which was made from an Australian wheat and contained about 30 per cent wheat bran was used in the experiments described below. A Canadian wheat was also useful but gave slight differences in the results at each step of the method. Other wheat germ may also be used.

Assay Method—See the first report (*1*).

Preparation of Resin—See the first report (*1*).

Purification and Crystallization of Wheat Cytochrome c

Step 1. Extraction—Two kg. batches of wheat germ were put into a glass column (7 × 160 cm.) and washed with ethylacetate which was circulated through the germ layer by evaporation and condensation for several hours using an apparatus alike to Soxhlet's extractor. Eleven kg. of the germ was washed in this way with ethylacetate, and then the solvent was evaporated off at room temperature. The washed and dried germ (10 kg.) was suspended in 50 liters of 30 per cent saturated AS** solution and allowed to stand overnight in a cold room (*ca.* 10°). After addition of 2 kg. of coarse celite (Celite 545), the suspension was squeezed through a thick cloth in a press. The residue was resuspended

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** AS represents ammonium sulfate.

in 20 liters of 30 per cent saturated AS and treated as above. The combined extracts (a slightly turbid, dark brown solution, of *ca.* 50 liters, at pH 6.5) were filtered in a Buchner funnel with the aid of 500 g. of Hyflo Super Cel and the residue was washed with 1 liter of 30 per cent saturated AS solution (combined filtrate, of *ca.* 50 liters, 30 per cent saturated with AS, at pH 6.5).

Step 2. AS Precipitation and Acid Treatment—To the filtrate obtained at the preceding step solid AS was added at the rate of 460 g. per liter of solution. The mixture was kept at room temperature (*ca.* 15°) for 2 or 3 hours with occasional stirring. The heavy precipitate formed was collected with 1 kg. of Hyflo Super Cel on a large Buchner funnel and washed with about 5 liters of 95 per cent saturated AS. The washed cake was squeezed in a cloth to drain out the AS solution (*ca.* 3 kg., containing roughly 0.8 kg. of AS). It was suspended in 6 liters of water, and then 4 liters of 20 per cent saturated AS was added to the thick suspension. After standing at room temperature for a few hours, the suspension was chilled to below 5°. Glacial acetic acid was added with stirring until the pH was reduced to 4.5, and the suspension was then kept at 5° for 50 minutes. After addition of 500 g. of Hyflo Super Cel, the suspension was filtered through a Buchner funnel and the residue on the funnel was washed with 5 liters of 20 per cent saturated AS solution of pH 4.5. The combined filtrate and washings (pH 4.7) was immediately adjusted to pH 6.5 with 30 per cent ammonia (a dark brown solution, of *ca.* 14 liters, at pH 6.5, 20 per cent saturated with AS).

Step 3. AS Fractionation—To the solution obtained at Step 2 was added solid AS until the specific gravity of the solution became 1.192 (*ca.* 75 per cent saturated). The resulting suspension was allowed to stand at room temperature for 1 hour. It was filtered through a Buchner funnel with the aid of 200 g. Hyflo Super Cel and the residue on the funnel was washed with 2 liters of 75 per cent saturated AS. (The liquid was pressed out of this residue and it was used as the source material for two kinds of crystalline hemoproteins having α-bands at 556 and 566 mμ (*9*, *10*)). To the combined filtrate and washings, solid AS was added to a specific gravity of 1.240 (*ca.* 95 per cent saturated), and then the suspension was adjusted to pH 7.5 by the addition of a small amount of 30 per cent ammonia. After standing over-night at room temperature, the precipitate which contained all of cytochrome c was recovered by filtration with 20 g. of Hyflo Super Cel on a Buchner funnel and washed with 300 ml. of saturated AS solution. The precipitate was suspended in 800 ml. of water, and insoluble matter was removed with a small Buchner funnel, and washed with 300 ml. of water (combined filtrate and washing, clear brownish red *ca.* 1 liter).

Step 4. Dialysis—The solution was dialyzed against tap water over-night at room temperature, and then against 10 liters of distilled water for several hours in the cold room. The brown precipitate which appeared during dialysis, was removed on a Buchner funnel with the aid of 20 g. Hyflo Super Cel and washed with 200 ml. of distilled water (combined filtrate and washing, clear brownish pink, *ca.* 1.5 liters).

Step 5. First Adsorption and Elution—Amberlite XE-64 was equilibrated with 0.05 *N* (0.05 g. ion of NH_4^+) ammonium phosphate buffer of pH 7.0. A resin column of 20 cm. height was made from it in a tube. The dialyzed cytochrome c solution obtained at Step 4 was passed through the column, and the column was washed with 500 ml. of the 0.05 *N* buffer. The upper larger part of the resin column which became gray (on which impure proteins which have higher affinity to the resin than cytochrome c were adsorbed (see RESULTS AND DISCUSSION)) was carefully removed. Then the red colored band (where the cytochrome c was adsorbed) was separated from the remaining part of the resin (which was only slightly colored). This red portion was resuspended and placed on the fresh resin (3 cm. high) in another column of 2 cm. in diameter. Thus a resin column of about 10 cm. in height was made. A large volume of 0.01 *N* buffer containing 0.001 *M* potassium

ferricyanide was passed through the column until cytochrome c adsorbed on the upper part of resin column had moved onto a lower part of the fresh resin and a little eluted from the column. The cytochrome c remaining on the column was then eluted with 0.3 *N* buffer of pH 7.0 (eluate, clear pink, *ca.* 30 ml., containing *ca.* 0.25 *N* NH_4^+, pH 6.6).

Step 6. Second Adsorption and Elution—The eluate was diluted three-fold with distilled water and passed through a column (2×10 cm.) of resin equilibrated with 0.1 *N* buffer of pH 7.0. Then the column was washed with the same buffer until about 5 per cent of the total cytochrome c adsorbed on the column had been eluted. The cytochrome c remaining on the column was then eluted with 0.25 *N* buffer of pH 7.0 (second eluate, reddish pink, *ca.* 40 ml., slightly less than 0.25 *N* NH_4^+, pH 6.8).

Step 7. Chromatography in Oxidized State—The second eluate was diluted 1.7-fold with distilled water, passed through a column (2×25 cm.) of resin equilibrated with 0.15 *N* buffer of pH 7. The cytochrome c adsorbed on the top of the column was developed with the same buffer. The effluent was collected in 3 ml. fractions around main part, and in 20 ml. fractions for other. Fractions having a high extinction ratio, R_{280}^{550}, of 0.8 or more were combined (clear pink, *ca.* 150 ml.).

Step 8. Concentration—The same as step 6 of Method (A) of the preceding report (*4*) except that 0.3 *N* buffer was used for elution (resulting solution, dark red, *ca.* 5 ml.).

Step 9. Chromatography in Reduced State—A concentrated solution of cytochrome c was reduced at pH 8.5 with ascorbic acid. It was chromatographed in a similar way to Step 7 of Method A of the preceding report (*4*) using a 2×25 cm. column and 0.125 *N* (0.125 g. ion NH_4^+) buffer of pH 7.0 containing 0.001 *M* ascorbic acid (main effluent, *ca.* 250 ml.).

Step 10. Concentration—The reduced cytochrome c in the main effluent of Step 9 was adsorbed on a small resin column and eluted with 10 per cent saturated AS of pH 8 as in Step 6 of Method I (*1*) (concentrated solution, *ca.* 1 ml.).

Step 11. Crystallization—To the concentrated reduced cytochrome c were added a small drop of 30 per cent ammonia and a few mg. of ascorbic acid. Then powdered AS was added until the solution became slightly turbid (see Step 7 of Method I (*1*)). A slight precipitate formed was removed by centrifugation at 8,000×*g* for 10 minutes. Then a little more AS was added to the supernatant. The slightly turbid solution was allowed to stand at room temperature (15–25°), and long plate shaped crystals usually appeared in a few days. If a suspension of these crystals was kept in the cold room (6–10°) for a few months further, the mother liquor became almost colorless and complete crystallization of cytochrome c occurred. Recrystallization was carried out in the same way, after dissolving the crystals in a minimum amount of water. A photograph of the crystals is shown in Fig. 2.

RESULTS AND DISCUSSION

Extraction of Cytochrome c from Wheat Germ—For the extraction of cytochrome c from wheat germ Goddard (*7*) used two methods: one with 4×10^{-3} *M* acetic acid at 80°, and the other with 0.1 *M* potassium dihydrogenphosphate after treatment of the germ with acetone, benzene and the mixture of benzene and ether successively. The cytochrome c of wheat germ did not loose its typical absorption spectrum by the heat treatment, but it was greatly modified like animal cytochrome c. Therefore, extraction at high temperature should be avoided. Since cytochrome c was not extracted efficiently in the cold, even by treatment of the germ with acetone or ether, treatment with some other organic solvents was examined. Better

results were obtained with ethyl acetate, which was also useful for the extraction of the pigment from yeast (*5*). For extraction from wheat germ treated with the organic solvent, 10–20 per cent saturated AS at pH 6.5 seemed to give the highest yield, though the squeezing out procedure was very difficult with this solution. When 30 per cent saturated AS solution was used for the extraction, the squeezing out procedure was much easier, though considerable amounts of filter aid (Celite 545) were still needed. Representative results of the above experiments are summarized in Table I.

TABLE I

Extraction of Cytochrome c from Wheat Germ

Pretreatment	Extraction	Amount of cyt. c*
None	Water	
None	0.1 *M* phosphate, pH 6.5	+
None	20% sat. AS, pH 6.5	++
None	30% sat. AS, pH 6.5	++
Acetone	30% sat. AS, pH 6.5	++
Alcohol	30% sat. AS, pH 6.5	++
Alc.+Et. acetate	30% sat. AS, pH 6.5	++
Et. acetate	Water	++
Et. acetate	0.1 *M* phosphate, pH 6.5	++
Et. acetate	20% sat. AS, pH 6.5	+++
Et. acetate	30% sat. AS, pH 7.5	++
Et. acetate	30% sat. AS, pH 6.5	+++
Et. acetate	30% sat. AS, pH 6.0	++

* Amounts of cytochrome c extracted were compared spectroscopically after reduction with sodium dithionite.

Precipitation of Cytochrome c—Keilin and Hartree (*12*) purified wheat germ cytochrome c by precipitation with trichloroacetic acid in concentrated AS. This method removed impurities from the wheat cytochrome c effectively and, by using this together with resin treatments, the pigment was crystallized. However, even more of the pigment was modified by the trichloroacetic acid-AS procedure than in the case of animal cytochrome c's. Also there was a low yield of the crystalline material. When the pigment was fairly pure, wheat cytochrome c was difficult to precipitate completely even with saturated AS. When the solution contained much foreign protein it was easily precipitated with AS together with the impurities. Precipitation at a slightly alkaline pH was easier than from a more acidic solution, and most of the pigment was precipitated between 80–90 per cent saturation of AS at pH 7.5.

Adsorption and Chromatography on the Resin—Wheat and animal cytochrome c can be adsorbed on neutral Amberlite XE-64 from a neutral solution of

low salt concentration, but the salt concentration must be lower for the wheat cytochrome c. When the dialyzed solution at Step 4 was passed through a column of Amberlite XE-64 with 0.05 *N* buffer of pH 7.0, large amounts of impurities which have higher affinity to the resin than cytochrome c were adsorbed on the upper part of the column and most of cytochrome c formed a distinct band below this brownish portion, which became grey after washing with a large volume of 0.05 *N* buffer. A little cytochrome c was usually found to remain with the impurities in the upper portion but this was thought to be in a modified form. Therefore, this upper part was cut off together with the grey part, aud only the part of the resin corresponding to the main band of cytochrome c was transferred to another column. During washing with 0.01 *N* buffer at Steps 5 and 6, even when the main band still remained on the column some cytochrome c was eluted together with much impurity. This eluted portion which was thought to be combined with some impurity, was rejected. When the band of cytochrome c approached the end of the column, a blue colored protein having an adsorption band at 618 mμ (presumably a copper protein) was eluted, together with small amount of cytochrome c. This eluate was also rejected. The blue protein also appeared with the initial main cytochrome c effluent on chromatography in Step 7. Since wheat germ contained many impurities having similar adsorption characteristics to cytochrome c, the pigment was not sufficiently purified by a single chromatography in the

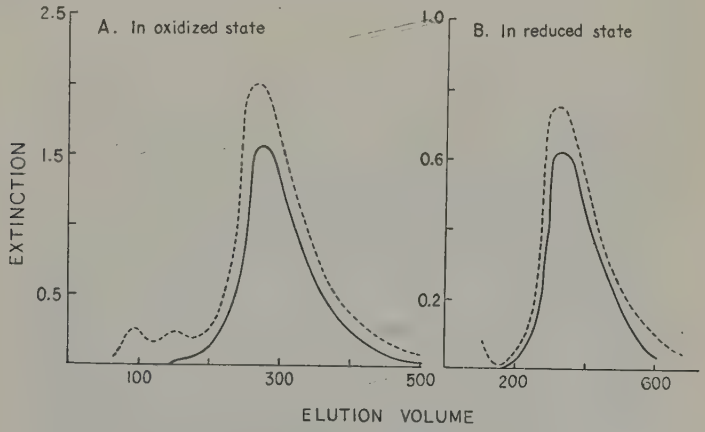

FIG. 1. Chromatography of wheat germ cytochrome c.
Column; Amberlite XE-64, 2×25 cm. Buffer; 0.15 *N*, pH 7.0 for A, 0.125 *N*, pH 7.0 for B. Solid line; extinction at 550 mμ. Dotted line; extinction at 280 mμ.

oxidized state even after previous purification by the two careful adsorption and elution procedures. Therefore, as in the case of beef kidney (*4*) rechromatography in reduced state was necessary to obtain the crystalline preparation. However, the two chromatographic treatments were made with a lower buffer concentration, (0.15 and 0.125 *N*,) than in the case of beef

kidney. Representative results of the two chromatographic treatments are shown in Fig. 1.

TABLE II

Summary of Purification and Crystallization of Cytochrome c from Wheat Germ

Step	Volume (*ml.*)	E_{280}	$E^{red.}_{550}$	E^{280}_{550}	Yield (*μ moles*)
(Wheat germ)	(9 *kg.*)				
Extraction	50,000	50	slight		
As fractionation, acid treat.	12,000	80	slight		
2nd AS frac. (75–95% sat.)	1,000	120	0.690	0.006	25*
Dialysis and filtration	1,500	27	0.411	0.015	22*
Adsorp. and frac. elution	28	23	8.16	0.35	8.2
2nd adsorp. and frac. elution	41	9.2	4.60	0.50	6.8
Chroma. in oxidized state	140	1.1	0.91	0.86	4.6
Chroma. in reduced state	240	0.50	0.46	0.92	4.0
Concentration by resin	1.0	110	101	0.92	3.6
Crystallization	0.65	130	130	1.0	3.0

* Values are not exact due to impurities.

Yield and Purity at Each Step—In Table II are shown representative results of yields and purities at each step of the present method. The yield of the crystalline cytochrome c was very low, being about 2.8 *μ* moles* per 10 kg. of wheat germ.

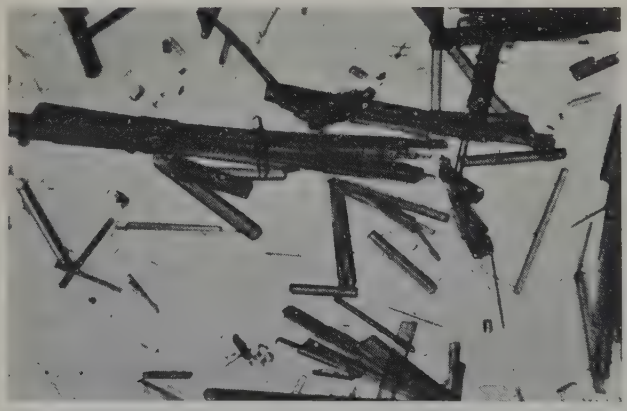

FIG. 2. Crystalline reduced cytochrome c of wheat germ (×200).

This yield may be increased if the extraction method is improved, since much cytochrome c still remained in the residue in the present method. Though many careful procedures were required in the present method,

* This value was estimated using molar extinction coefficient for beef heart cytochrome c, $\varepsilon = 2.77 \times 10^4\ M^{-1} \cdot cm^{-1}$.

crystallization was very easy when the pigment was sufficiently pure.

SUMMARY

Wheat germ was treated with ethylacetate and cytochrome c was extracted from it with 30 per cent saturated ammonium sulfate. It was then fractionated with AS. The precipitate between 75–95 per cent saturation was dialyzed, adsorbed on Amberlite XE-64, and eluted with 0.3 *N* NH_4^+ buffer of pH 7 after washing the column with 0.1 *N* buffer. The adsorption, washing and elution treatments were repeated, and then the cytochrome c was chromatographed first in the oxidized and then in the reduced state. The purified cytochrome c was then easily crystallized in a similar way to mammalian cytochrome c into long plates. The yield was about 3 μ moles per 10 kg. germ.

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AN IMMUNOCHEMICAL STUDY OF MICROBIAL AMYLASE. II

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Studies on the antigen-antibody reaction of enzyme have been reported by a number of workers (*1–3*), and we also reported the formation in rabbit of antibacterial-α-amylase antibody which inactivates the activity of crystalline bacterial amylase and some serological properties of this antibody (*4, 5*).

Since Taka-α-amylase, a mold enzyme, can be easily obtained in a pure state, determination of its activity is quite accurate, and its chemical composition and structure have been extensively studied. This enzyme seems, therefore, to be a suitable material for the studies of enzyme-immunochemistry.

Earlier immunological study by A m a n o (*6*) has demonstrated that when alum-precipitated crystalline Taka-α-amylase was injected to rabbits, the rabbits apparently produced antibody homologous to Taka-α-amylase. Although 4 times recrystallized Taka-amylase was used as antigen, two lines were observed in the precipitation in agar medium, indicating the inhomogeneous nature of crystalline Taka-amylase used. It was also noted that over 94 per cent amylase activity was neutralized with the homologous antibody.

The purpose of the present paper is to confirm the original experiment by A m a n o, and to extend the problem further from the enzymological stand point of view.

In the present research, the formation of antibody against Taka-α-amylase and serological and enzymological properties of this antibody were studied.

METHODS

Antigen—Lyophilized crystalline Taka-α-amylase, which was prepared from Taka-diastase Sankyo by A k a b o r i's method (*6*), and recrystallized 3 times with aceton solution containing 0.01 *M* calcium acetate, was used.

Antibody—Three rabbits weighing 2 kg. or more were injected intravenously with 1 ml. of 1 per cent (10 mg./ml.) saline solution of Taka-amylase. Six injections were given, one every other day, in the course of 11 days. The rabbits were sacrificed by bleeding from carotid artery 16 days after the last injection and sera were separated. The sera were inactivated at 56° for 30 minutes and stored in frozen state.

Methods for the preparation of antibacterial-α-amylase antibody, assay of the amylase activity, calculation of the percentage of the inhibited activity of amylase, and for determination of reducing sugar were described in the previous reports (*4*, *5*). Serological properties of anti-Taka-amylase antibody were studied by ring test, gel diffusion method (*7*), immunoelectrophoresis (*8*, *9*) and inhibition test of Taka-amylase activity.

RESULTS

Serological Properties of Anti-Taka-amylase—Ring test was performed using serial 2 folds dilution of anti-Taka-amylase antisera and 100 mg./ml. Taka-amylase solution as shown in Table I. The antibody titer of each of these

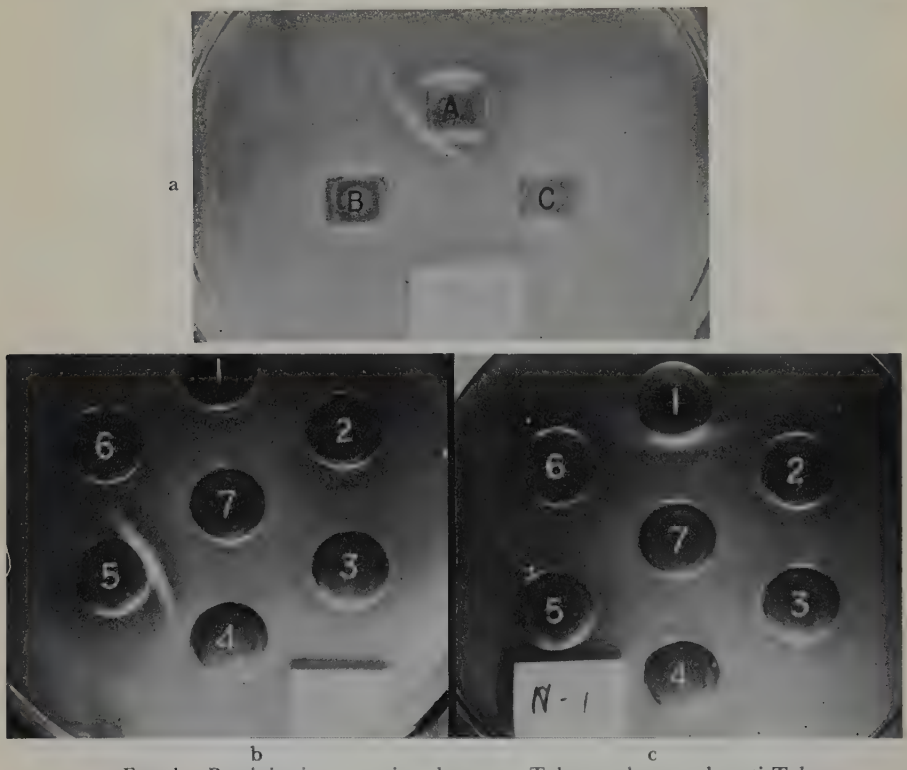


FIG. 1. Precipitation reaction between Taka-amylase and anti-Taka-amylase antisera (photograph a), anti-bacterial antisera (b), and anti-heat denatured bacterial amylase (c).

(a) Basin A, anti-Taka-amylase antiserum.
„ B, Taka-amylase solution (10 mg./ml.).
„ C, bacterial amylase (10 mg./ml.).

(b) Basin 1, anti-bacterial antiserum (No. 6649).
„ 2, „ „ (No. 6651).
„ 3, anti-heat denatured bacterial antiserum (No. 6652).
„ 4, „ „ (No. 6654).
„ 5, anti-Taka-amylase antiserum (No. 6670).
„ 6, „ „ (No. 6669).
Center 7, Taka-amylase (10 mg./ml.).

(c) Basins 1, 2, 3, 4, 5, 6 same as those of (a) center (7) bacterial amylase (10 mg./ml.).

sera was 1 : 16, 1 : 64 and one negative. The reaction between those sera and the bacterial-α-amylase was negative.

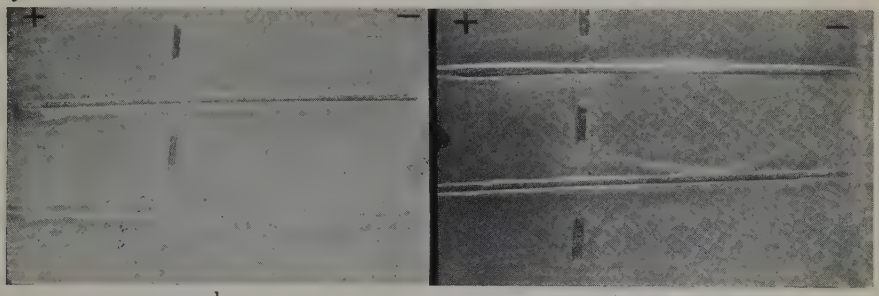
TABLE I

Precipitation Reaction of Anti-Taka-amylase Antiserum

Batch No. of Taka-antiserum	Serum dilution 1×2^n n=										
	0	1	2	3	4	5	6	7	8	9	10
6668	0	0	0	0	0	0	0	0	0	0	0
6669	2	2	1	1	1	?	0	0	0	0	0
6670	3	3	3	2	2	1	1	?	0	0	0

Result of ring test after 4 hours.
100 μg./ml. Taka-amylase solution was used as antigen.

Results of gel diffusion method (Ouchterlony method) were shown in Fig. 1. Only one precipitation line was observed between Taka-α-amylase and its antiserum, which suggests that the Taka-amylase is composed of a single antigenic component.



d e

FIG. 2. Immunoelectrophoresis of Taka-amylase and bacterial amylase (by Graber). Photographs (d, e).

Medium; 2 per cent agar in pH 8.4 1/10 *M* barbitar buffer. Antigen; 30 mg./ml. Taka-amylase added in basins 1 and 2. Electrophoresis was carried out at 250 volt, 8 mA and 3 hours. After electrophoresis antiserum was added into basin 3.

(d): Precipitation line between Taka-amylase and its antiserum on the anodic side.

(e): Precipitation line between bacterial amylase and its antiserum on the cathodic side.

The reaction between bacterial-amylase and anti-Taka-amylase antiserum was negative, and also no precipitation line was observed between Taka-amylase and anti-bacterial-α-amylase antiserum. The above findings suggest that there is no cross reaction between Taka-amylase and bacterial-amylase.

Taka-amylase was also examined immunoelectrophretically (Fig. 2).

Results also suggest that Taka-amylase is composed of a single antigenic component. Furthermore, Taka-amylase showed the different electrophoretic mobility from that of bacterial-α-amylase, the former moved to cathodic side but the latter moved to anodic side.

Inhibition of Enzymatic Activity of Taka-amylase by Its Antibody—The inhibition of Taka-amylase activity by anti-Taka-amylase antiserum was investigated. Results were essentially similar to those of bacterial-amylases.

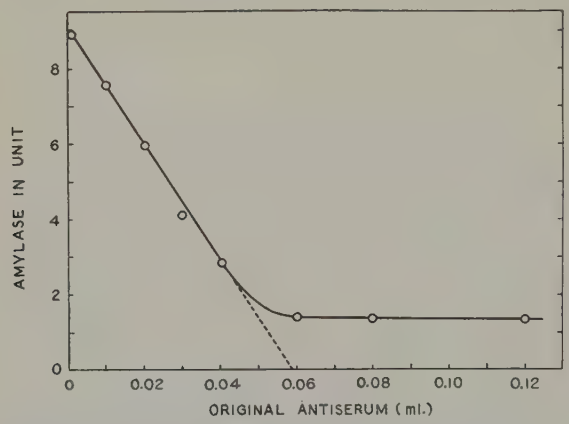


FIG. 3. Inhibition curve of Taka-amylase with its antiserum.

0.4 ml. of Taka-amylase solution (8.5 unit) was mixed with its antiserum. Enzyme activity was assayed after the incubation of 1 hour at 40°.

TABLE II

Effect of Various Carbohydrates on the Amylase-Anti-amylase Reaction

Carbohydrate	Concentration of carbohydrate	% inhibition
Glucose	5	0
Lactose	5	0
Maltose	5	10
Isomaltose	5	12
Panose	5	12
Maltotriose	5	32
Dextran	5	0
Residual dextrin	5	26
Starch hydrolysate	10	60

0.4 ml. of 40 μg./ml. Taka-amylase solution and 0.2 ml. of 20 fold diluted antiserum were mixed with 0.1 ml. of 5 per cent solution of various carbohydrates and filled up with calcium acetate solution to 1 ml. After 60 minutes, residual amylase activity was measured.

As shown in Fig. 3, a linear relationship was obtained between degree of inhibition and quantity of antiserum used, but complete inhibition of the enzyme activity was not attained.

Effect of Starch and Various Carbohydrates on the Taka-amylase Anti-Taka-amylase System—Inhibition of amylase-antiamylase reaction by substrate, starch, and its degradated products, various carbohydrates was examined. The results were summarized in Table II and Fig. 4. As shown in Fig. 4, the apparent competitive interaction between starch or its degradated products and antibody was observed, and it became clear that the starch hydrolysate had the strongest effect on the antigen-antibody reaction.

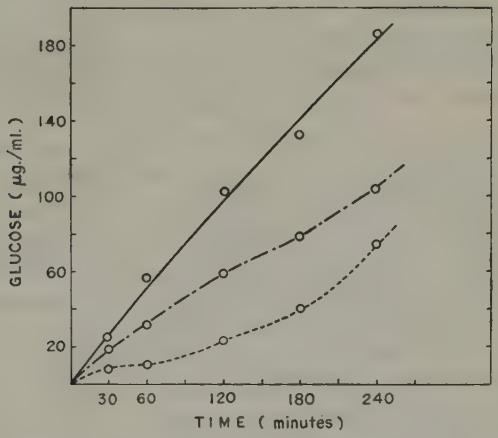


FIG. 4. Effect of starch on the Taka-amylase anti-Taka-amylase reaction.

Effect of the addition of anti-Taka-amylase before and after amylase action has started on the soluble starch as a substrate. —○—; 0.5 ml. of amylase solution (1 μg. of amylase) and 0.5 ml. of 0.85 per cent NaCl were incubated at 40°. After 35 minutes, 1 ml. of 2 per cent starch was added. —•○—•; 0.5 ml. of amylase solution and 1 ml. of 2 per cent starch were mixed. After 5 minutes, 0.5 ml. of 200-fold diluted antiserum was added. – –○– –; 0.5 ml. of amylase solution and 0.5 ml. of 200-fold diluted antiserum were mixed. After 35 minutes, 1 ml. of 2 per cent starch solution was added. The liberation of reducing substances (experssed as glucose) was followed colorimetrically.

The Reaction of Anti-Taka-amylase on Various Amylases of Other Origins—Results of the reaction between anti Taka-amylase and various amylases of other origins are shown in Tables III, IV, V. Anti-Taka-amylase antisera were found to inhibit the activity of partially purified α-amylase of Aspergillus species as well as that of Taka-amylase obtained from *Aspergillus oryzae*, but it did not inhibit β-amylase activity of *Aspergillus usami* and *Aspergillus niger*. Furthermore, this antiserum had no inhibitory effect to α, β-amylase of other origins tested.

The Effect of the Heat Denatured Taka-amylase on the Anti-Taka-amylase—Heat

TABLE III

The Effect of Anti-Taka-amylase on Various α-Amylases of Different Origins

Origins	Amylase unit		% inhibition of amylase activity
	Enzyme	Enzyme+antisera	
Taka-α-amylase	9.4	2.1	77
Asp. usami α-amylase	8.6	2.1	75
Malt-α-amylase	6.4	7.0	0
Bac. subtilis N α-amylase	6.4	6.5	0

0.4 ml. of antigen solution (concentration of enzyme solution was 10–5 units as amylase activity) was mixed with 0.2 ml. of antiserum (20-fold dilution) filled up with 0.85 per cent NaCl to 1 ml. and after 60 minutes remaining amylase was measured.

TABLE IV

The Effect of Anti-Taka-amylase Antiserum on Various α-Amylases of Molds

Origins	Amylase unit		% inhibition of amylase activity
	Enzyme	Enzyme+antisera	
Asp. sojae	2.9	1.8	38
Asp. oryzae	4.4	2.7	40
Asp. awamori	4.7	2.1	56
Asp. usamii	4.3	2.0	53
Rh. tonkinensis	3,8	3.8	0
Rh. peka	1.5	1.5	0
M. spinescens	2.3	2.3	0
Pen. chrysogenum	0.8	0.8	0

Experimental conditions were the same as those described in Table III. These α-amylases of molds were partially purified.

TABLE V

The Effect of Anti-Bacterial (subtilis N)-α-Amylase on the Various α-Amylase of Different Origins

Origins	Amylase unit		% inhibition of amylase activity
	Enzyme	Enzyme+antisera	
Bac. subtilis N amylase	4.86	2.76	43
Asp. usami α-amylase	4.86	4.86	0
Malt α-amylase	5.21	5.70	0
Taka-amylase	7.50	7.50	0

Experimental conditions were the same as those described in Table III.

denatured Taka-amylase (100°, 20 minutes) did not inhibit the reaction between Taka-amylase and its antiserum.

DISCUSSION

The antisera against Taka-amylase were prepared by immunization of rabbits with the recrystallized enzyme. From the serological examinations it became clear that this antiserum contains a single homogeneous antibody with a moderate high activity.

From the facts that the anti-Taka-amylase antibody inhibits enzyme activity of Taka-amylase and that this reaction is competitively inhibited by starch and its degradated products, the antigen-antibody interaction appears to mask the enzymatically active sites of the enzyme molecule, as in the case of the amylase of *Bacillus subtilis* and its antibody. The two amylases, however, can be distinguished serologically because cross reaction between one enzyme and heterologous antibody dose not occur. Moreover, of all the amylase preparations from the various origins tested, only α-amylase of Aspergillus genus was inhibited by anti-Taka-amylase antiserum.

Crystalline Taka-amylase has almost the same enzymological specificity with crystalline-α-amylase from malt, *Bac. subtilis* and animal origins, although serological activity, electrophoretic mobility, solubility and pH tolerance of those enzymes differ markedly from each other. The striking differences in the serological behaviors of α-amylase described suggest the difference in the structures of those amylase molecules. This was further supported from the observation that the denatured Taka-amylase does not react with the antiserum in accordance with the pioneer work of Roy and Chakravarty (*10*). Although anti-Taka-amylase antibody specifically inhibits the activity of the α-amylases from Aspergillus species, the identity of the active site of α-amylases from various origins can not be determined. Since the modification of the structure of the enzyme by the antisera may possibly affect the enzymatic activity of the molecule. The immunochemical specificity of various amylases reported here may provides a clue for the classification of microorganisms.

The activity of Taka-amylase was decreased by the antisera but the complete inactivation could not be attained. The same phenomenon has been observed with anti-bacterial-α-amylase antisera. This might be explained either by the reversible dissociation of antibody-enzyme complex or by assumption that the complex exhibits an amylase activity.

SUMMARY

1. Antisera against Taka-α-amylase were produced in rabbits by the injection of crystalline Taka-α-amylase.

2. Serological properties of antisera obtained were studied and it became clear that the antisera contain only a single homogeneous antibody with a moderate high activity.

3. The inhibition of Taka-amylase activity by antibody, and the inhibitory effect of starch and other carbohydrates on the amylase-antiamylase reaction, were clearly demonstrated.

4. The effect of an anti-Taka-amylase antibody on the various amylases of other origins was studied. It was found that anti-Taka-amylase antiserum inhibits the α-amylase activities of *Aspergillus usami*, *awamori* and *sojae*; on the other hand the α-amylase activities of other molds and β-amylase activities of all strains tested were unaffected.

5. Heat denatured Taka-amylase did not inhibit the reaction between the Taka-amylase and its antiserum.

The author is indebted to Prof. S. Akabori for his encouragement and guidance, and to Prof. T. Ogata, in whose laboratory the immunization experiment was perfomed, for his helpful advices and suggestions, especially on the immunological technique. The author also wishes to thank Akabori laboratory, University of Osaka, for generous supply of crystalline Taka-α-amylase and to Dr. Ando for his kind gifts of mold α-amylase preparations.

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ACTION OF CHYMOTRYPSIN ON SYNTHETIC SUBSTRATES

II. ACTION OF α-CHYMOTRYPSIN ON AMINOACYL-L-TYROSINE ETHYL ESTERS

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(Received for publication, September 6, 1958)

Studies on the specificity of chymotrypsin have demonstrated that the enzyme hydrolyzes peptide linkages of tyrosyl and phenylalanyl peptides which involve the carbonyl residue of the aromatic amino acids (*1*). Subsequent studies have shown that this enzyme hydrolyzes the ester bond of acylamino acid ester having general formula R′CO-NH(CHR)CO-OR″. In the substrates tested, the group R′CO- was acetyl or benzoyl for example, admittedly unphysiological substituents (*1*). No acylamino acid ester in which the acyl substituents are amino acid goups have been reported to be subjected to the action of chymotrypsin.

In the preceding paper of this series, a number of aminoacyl-L-tyrosinamide have been tested as substrates for chymotrypsin (*2*). In the present study, a number of aminoacyl-L-tyrosine ethyl esters in which the aminoacyl substituents are the aliphatic amino acid groups were prepared, and were subjected to the action of α-chymotrypsin.

EXPERIMENTAL

Enzyme—The preparation of α-chymotrypsin was the same as that previously described (*2*).

Measurement of the Extent of Disappearance of Ester—The method used to follow the disappearance of ester was based on the hydroxamic acid method of Hestrin (*3*), and the procedure of Wiggans *et al.* (*4*) was slightly modified as follows. In the case of 0.01 *M* substrate concentration, 0.4 ml. sample of the incubation mixture was diluted with 1 ml. of a 1 : 1 mixture (*v/v*) of 3.4 *N* NaOH and 2 *M* hydroxylamine hydrochloride. After 4 minutes at room temperature, 0.5 ml. of 3.2 *N* HCl and 0.5 ml. of 0.74 *M* $FeCl_3$ in 0.1 *N* HCl were added with shaking. The optical density was read in Hitachi electric photometer (EPO-A), using 530 mμ filter. The blank determination was carried out in the same way with 0.4 ml. of water. For each ester, a standard curve was prepared, relating extinction to concentration of ester, thus permitting the calculation of per cent desappearance of ester.

Measurement of the Extent of Hydrolysis of Ester (Formation of Dipeptide)—In general, the reactions in the presence or absence of the enzyme occur as follows:

dipeptide ester → dipeptide + ninhydrin negative product (I)

The extent of the hydrolysis of the substrate to form free dipeptide was determined by the colorimetric ninhydrin method, using the eqation (III) which is derived as follows. Since an incubation mixture contains dipeptide ester and free dipeptide as ninhydrin positive substances, which are shown on the paper chromatography* (Table I), and ninhydrin negative products, the optical density of a sample drawn at any interval of time may be represented as follows:

$$D_t=\frac{\alpha(a_o-a)+\beta b}{\alpha a_o}\times D_o \qquad \text{(II)}$$

TABLE I

R_f Value of the Compounds[1]

The compounds were chromatographed on Toyo Roshi No. 50 paper. The ascending technique was applied.

Substance	R_f in *n*-butanol : acetic acid : pyridine : water	
	15 : 3 : 10 : 12 (*v/v*)	4 : 1 : 1 : 2 (*v/v*)
GlyTyrOEt	0.73	0.71
AlaTyrOEt	0.79	0.76
ButTyrOEt	0.84	0.81
NleuTyrOEt	0.90	0.89
β-AlaTyrOEt	0.71	0.71
ValTyrOEt	0.86	0.86
LeuTyrOEt	0.91	0.91
ε-NleuTyrOEt	0.81	0.75

1) The R_f values of the parent dipeptides, aminoacyl-L-tyrosines, have been reported in previous communication (*2*).

In this equation, a_o is the initial ester concentration, a is the disappearing ester concentration at t minutes, b is the forming dipeptide concentration, D_o and D_t are the optical density of samples at 0 and t minutes, α and β are the color yield of dipeptide ester and dipeptide respectively, based on L-leucine as 100 per cent. The optical density was measured in the same procedure as that described in the previous communication (*5*, *6*). From equation (II) the following relationship is derived:

$$\%\ \text{hydrolysis or dipeptide formation}=\frac{\alpha}{\beta}\times\left(\%\ \text{ester disappearance}-\frac{D_o-D_t}{D_o}\times 100\right) \qquad \text{(III)}$$

* At the same time, the possibility of the occurrence of transpeptidation reaction in the presence of the enzyme was tested by means of paper chromatography. In all cases, the apparent formation of free dipeptide was revealed on the chromatogram, while additional very faint spot of an unknown substance which might be produced by the transpeptidation reaction was recognized in the cases of Ala-, But-, Nleu- and Leu-TyrOEt. Since it seems that the unknown substances produced are the negligibly small in their amount, the equation (III) may be applied satisfactorily for all reactions tested in this study.

The color yields of dipeptide esters and corresponding dipeptides are shown in Table II.

TABLE II

Per Cent Color Yield of the Compounds Based on L-*Leucine* = 100%[1,2]

Experimental details are described in the literatures (*6*, *8*).

Substance	Color yield (*per cent*)	Substance	Color yield (*per cent*)
GlyTyr	93	GlyTyrOEt	92
AlaTyr	152	AlaTyrOEt	153
ButTyr	146	ButTyrOEt	147
NleuTyr	117	NleuTyrOEt	112
β-AlaTyr	73	β-AlaTyrOEt	59
ValTyr	63	ValTyrOEt	77
LeuTyr	112	LeuTyrOEt	108

1) The synthesis of aminoacyl-L-tyrosines has been described in the communication from this laboratory (*7*).

2) The color yields were obtained on heating for 15 minutes at 100°, using 570 mμ filter. The variation of the experimental conditions yielded the marked difference in the values of the color yields (*8*).

Synthesis of Substrates—L-*Alanyl-* and β-*alanyl-*L-*tyrosine ethyl ester hydrochloride* (*AlaTyrOEt HCl* and β-*AlaTyrOEt HCl*) were prepared as described previously (*4*, *9*).

*Glycyl-*L-*tyrosine Ethyl Ester Hydrochloride* (*GlyTyrOEt HCl*)—A simple method for the preparation of this compound was devised as follows. Tritylglycyl-L-tyrosine ethyl ester was prepared by the mixed anhydride method in the same manner as that for tritylglycyl-L-tyrosine benzyl ester (*5*, *10*). The crude crystals thus obtained were recrystallized from ethanol-petroleum ether. Yield, 76 per cent; m.p. 155–157°; $[\alpha]_D^{27}$ +23.4° (c 2, in ethanol).

$C_{32}H_{32}O_4N_2$ (508.6): Calcd. N 5.5
Found N 5.5

A solution of the above ester (10.2 g.) in 0.5 *N* methanolic HCl (42 ml.) was heated for 2 minutes in a water bath. Evaporation *in vacuo* and trituration of the residue with ether resulted in crystallization of GlyTyrOEt HCl. It was recrystallized from ethanol-ether. Yield, 5.6 g.; m.p. 234–235°; $[\alpha]_D^{27}$ +18.1° (c 2, in water).

$C_{13}H_{19}O_4N_2Cl$ (302 8): Calcd. N 9.3
Found N 9.4

This compound has been obtained previously by hydrogenolysis of carbobenzoxyglycyltyrosine ethyl ester; $[\alpha]_D^{22}$ +17.1° (*9*).

L-α-*Amino-n-butyroyl-*L-*tyrosine Ethyl Ester Hydrochloride* (*ButTyrOEt HCl*)—The carbobenzoxy-L-aminobutyroyl-L-tyrosine ethyl ester (0.003 mole) (*2*) dissolved in a mixture of methanol (10 ml.) and 0.5*N* methanolic HCl (6.6 ml.) was treated with dry hydrogen in the presence of palladium black. The filtrate from the catalyst was evaporated *in vacuo*, and the residual syrup was crystallized easily. It was recrystallized from methanol-ether, and dried *in vacuo* over P_2O_5 at 61°. Yield, 78 per cent; m.p. 112–113°; $[\alpha]_D^{20}$ +18.2° (c 2, in water).

$C_{15}H_{23}O_4N_2Cl \cdot H_2O$ (348.8): Calcd. C 51.6, H 7.2, N 8.0
Found C 52.1, H 7.4, N 7.9

L-*Norvalyl*-L-*tyrosine Ethyl Ester Hydrochloride* (*NvalTyrOEt HCl*)—The compound was prepared from carbobenzoxy-L-norvalyl-L-tyrosine ethyl ester (*2*) in the same manner as that for ButTyrOEt HCl. Yield, 82 per cent; m.p. 94–96°; $[\alpha]_D^{13}+13.8°$ (c 2, in water).

$C_{16}H_{25}O_4N_2Cl \cdot H_2O$ (362.9): Calcd. C 52.9, H 7.5, N 7.7
Found C 52.9, H 7.5, N 8.0

L-*Norleucyl*-L-*tyrosine Ethyl Ester Hydrochloride* (*NleuTyrOEt HCl*)—This compound was obtained from the carbobenzoxy derivative (*2*) in hygroscopic crystalline state. Yield, 77 per cent; m.p. 80–85°; $[\alpha]_D^{20}+5.1°$ (c 2, in water).

$C_{17}H_{27}O_4N_2Cl \cdot 1/2H_2O$ (367.9): Calcd. C 55.4, H 7.7, N 7.6
Found C 55.5, H 8.0, N 7.7

L-*Valyl*-L-*tyrosine Ethyl Ester Hydrochloride* (*ValTyrOEt HCl*)—The compound was prepared from the carbobenzoxy derivative (*2*). Despite repeated precipitation from methanol-ether, it could not be obtained in a crystalline state. Yield, 81 per cent; $[\alpha]_D^{20}+19.4°$ (c 2, in water). The analysis was not satisfactory.

$C_{16}H_{25}O_4N_2Cl$ (344.8): Calcd. C 55.7, H 7.3, N 8.1
Found C 54.5, H 7.8, N 8.0

L-*Leucyl*-L-*tyrosine Ethyl Ester Hydrochloride* (*LeuTyrOEt HCl*)—The compound was obtained from the oily carbobenzoxy derivative (*11*) in non-crystalline powder. Yield, 81 per cent; $[\alpha]_D^{27}+6.2°$ (c 2, in water).

$C_{17}H_{27}O_4N_2Cl$ (358.9): Calcd. C 56.9, H 7.6, N 7.8
Found C 56.0, H 8.0, N 7.4

ε-*Amino-n-caproyl*-L-*tyrosine Ethyl Ester Hydrochloride* (ε-*NleuTyrOEt HCl*)—The compound was obtained from the carbobenzoxy derivative (*2*) in non-crystalline form. Yield, 91 per cent; $[\alpha]_D^{20}+2.5°$ (c 2, in water).

$C_{17}H_{27}O_4N_2Cl$ (358.9): Calcd. C 56.9, H 7.6, N 7.8
Found C 55.8, H 8.0, N 7.5

RESULTS AND DISCUSSION

pH-Activity Curve of GlyTyrOEt—For the comparison of the susceptibility of aminoacyltyrosine ethyl esters, it seems desirable to determine the rates of their disappearance around their optimum pH.

Comparative measurements of the effect of pH on the esterase activity of chymotrypsin were made with GlyTyrOEt as a representative substrate. At the pH tested, two possible non-enzymatic reactions may occur: spontaneous hydrolysis of ester and cyclization to form diketopiperazine. The first order velocity constants, $K_{no\ enz.}$, which are shown in Fig. 1, were determined by measurements of the disappearance of ester in the absence of added enzyme at various pH. In the same manner, the values of $K_{obs.}$ were obtained in the presence of the enzyme. Then, the first order velocity constants, $K_{enz.}$, were derived from $K_{obs.}-K_{no\ enz.}$. Since the disappearance of ester in the presence and absence of the enzyme did not follow exactly the kinetics of first order, the values of K at the zero time calculated graphically were taken for the comparison of sensibility of the substrate at the pH tested. The results were shown in Fig. 1, an optimum pH for the disappearance of ester by the enzyme appearing near 8.3.

In the previous paper (*2*), it was reported that an optimum pH of glycyltyrosinamide fell in the pH 8.3. It would be of interest to note that the optimum pH of benzoyltyrosine ethyl ester is reported to be near 7.8 (*12*), and this value is appreciably lower than that of GlyTyrOEt. This result may be explained by the fact that the rate of the reaction will depend on the concentration of available substrate at a given pH as described already (*5*, *13*).

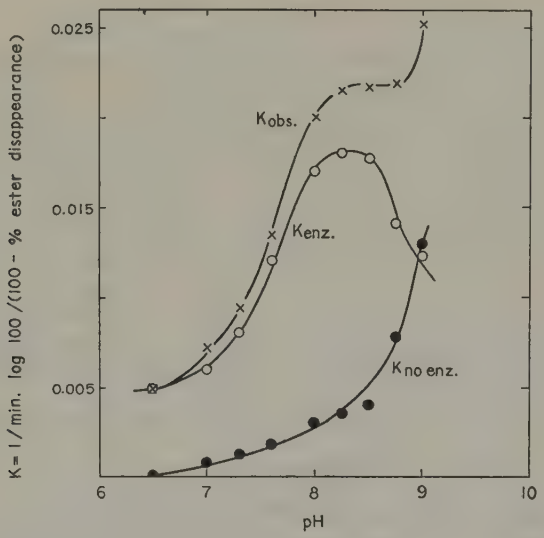

FIG. 1. The pH dependence of the disappearance of GlyTyrOEt in the presence and absence of chymotrypsin. The substrate concentration, 0.05 *M*; enzyme concentration, 0.26×10^{-3} mg. N per ml.; temperature, 30°. $K_{enz.} = K_{obs.} - K_{no\ enz.}$. Tris (0.1 *M*) was the buffer at pH 8.0~9.0; phosphate (0.1 *M*) at pH 6.5~8.0.

Disappearance of Esters in the Absence of Enzyme—Representative data for the disappearance and hydrolysis of GlyTyrOEt at 0.01 *M* compound concentration are shown in Table III as an example, the average value of $K_{no\ enz.}$ and $K_{hyd.\ no\ enz.}$ being listed in Table IV. As seen from Table III, it was found that the non-enzymatic disappearance of the substrates tested at 0.01 *M* followed approximately first order kinetics. Though the hydrolysis of GlyTyrOEt to produce the free peptide was occurred to some extent at 0.01 *M* concentration (Tables III and IV), no measurable amount of the hydrolysis could be observed at 0.05 *M* at pH 8.0* (Table IV). The values of velocity constants for other substrates are given in Table IV.

It should be noted that precipitates (diketopiperazines) appeared within about 10 hours in the case of 0.01 *M* GlyTyrOEt and within about 1~4 days in the case of the other compounds except β-Ala- and ε-Nleu-TyrOEt

* The presence of GlyTyr in very slight amount was recognized on the paper chromatogram after the standing of the solution for about 1 hour.

which produced no precipitates after standing the solution at pH 8.0 for a long time.

Table III

Example in Measurements of Disappearance and Hydrolysis of GlyTyrOEt in the Absence of Enzyme[1]

Compound concentration, 0.01 *M*; pH 8.0 (0.1 *M* phosphate buffer); temperature, 30°.

Disappearance (*per cent*)	Time (*min.*)	$K_{no\ enz.}$	Hydrolysis[2] (*per cent*)	Time (*min.*)	$K_{hyd.\ no\ enz.}$
3.4	2.9	0.0052	0.7	4.6	0.0007
5.8	5.8	0.0045	0.5	7.5	0.0003
8.2	8.8	0.0042	0	9.8	0
12.2	12.0	0.0047	1.6	14.3	0.0005
15.4	14.8	0.0049	1.9	17.1	0.0005
18.1	20.1	0.0043	0.9	20.4	0.0002
28.9	29.5	0.0050	4.1	40.5	0.0006

1) *K* denotes the first order velocity constant as defined in Bergman's work, *i.e.* $K=(1/t)\log(100/100-\%$ ester disappearance or hydrolysis). *t* is in minute.

2) Per cent of the hydrolysis was calculated from the equation (III).

Table IV

Velocity Constant of the Compounds in the Absence of Enzyme

Compound concentration, 0.01 *M*; pH 8.0 (0.1 *M* phosphate buffer); temperature, 30°.

Compound	$K_{no\ enz.}$	$K_{hyd.\ no\ enz.}$
GlyTyrOEt	0.0047	0.0004
„ [1]	0.0030	~0
AlaTyrOEt	0.0027	0.0005
ButTyrOEt	0.0006	~0
NvalTyrOEt	~0	
NleuTyrOEt	~0	
β-AlaTyrOEt	0	0
ValTyrOEt	0.0024	~0
LeuTyrOEt	~0	0
ε-NleuTyrOEt	0	

1) Compound concentration, 0.05 *M*.

Disappeanance of Esters in the Presence of Chymotrypsin—In order to compare the sensibility of the substrates to chymotrypsin, the values of proteolytic coefficients in respect with the disappearance and hydrolysis of esters were determined.

Representative data for GlyTyrOEt at 0.01 M concentration are given in Table V as an example. Since the first order reaction constants had the tendency to decrease slightly with increasing the time as seen from Table V, $K_{obs.}$ and $K_{hyd.\ obs.}$ at the zero time calculated graphically were taken for the comparison of sensibility of the substrate and the values were shown in Table VI. The values of K for other substrates are also given in Table VI.

TABLE V

Example in Measurement of Disappearance and Hydrolysis of GlyTyrOEt in the Presence of Chymotrypsin

Substrate concentration, 0.01 M; pH 8.0 (0.1 M phosphate buffer); enzyme concentration, 0.22×10^{-3} mg. N per ml.; temperature, 30°.

Disappearance (*per cent*)	Time (*min.*)	$K_{obs.}$	Hydrolysis[1] (*per cent*)	Time (*min.*)	$K_{hyd.\ obs.}$
15.9	2.1	0.036	17.1	3.4	0.024
33.2	5.0	0.035	31.8	7.0	0.024
46.2[2]	8.0	0.034	38.2	9.5	0.022
57.1	11.0	0.032	44.3	12.7	0.020
60.1	14.0	0.028	51.5	15.8	0.020
65.4	17.0	0.027	58.5	20.1	0.019
70.4	22.0	0.024	59.0	25.2	0.015
75.7	27.0	0.023			

1) Per cent of the hydrolysis was calculated from the equation (III).
2) A gelatinous precipitate began to appear at this point.

Of special interest was the finding that the gelatinous massive precipitates appeared within 2~8 minutes during the enzyme reaction in all cases except β-Ala- and ε-Nleu-TyrOEt. Though it may be surmised that the precipitates would be the diketopiperazines, they have not been identified yet. If diketopiperazines are produced during the enzyme reaction, chymotrypsin may prove to catalyze an intramolecular transpeptidation reaction to some extent, besides a hydrolytic reaction.

It will be seen from Table VI that β-AlaTyrOEt is solely hydrolyzed to β-AlaTyr, and no precipitate is observed even after standing the incubation mixture for a long time. The simple hydrolysis of the substrate is also demonstrated qualitatively by means of the paper chromatography at different intervals of time during the incubation. It seems that β-AlaTyrOEt is proved to be one of the typical excellent synthetic substrates for chymotrypsin because of its high susceptibility to the enzyme, the resistance to non-enzymatic hydrolysis and the easiness in synthetic procedure. The reaction of ε-Nleu-TyrOEt by chymotrypsin may be also a simple hydrolysis as that for β-AlaTyrOEt. It would be of interest in this connection to note β-AlaPhe-OEt is resistant to action of cathepsin C (*4*).

TABLE VI

Proteolytic Coefficient in Disappearance and Hydrolysis of the Substrates by Chymotrypsin

Substrate concentration, 0.01 *M*; pH 8.0 (0.1 *M* phosphate buffer); temperature 30°.

Substrate	Enzyme concn. (10^{-3} *mg. N per ml.*)	$K_{obs.}$ / $K_{enz.}$ [1]	$K_{hyd.\ obs.}$ / $K_{hyd.\ enz.}$ [2]	C [3]	$C_{hyd.}$ [4]
GlyTyrOEt	0.22	0.036 0.031	0.025 „	141	114
„ [5]	0.26	0.020 0.017	0.0012 „	65	4.6
AlaTyrOEt	0.90	0·028 0.025	0.019 0.018	28	20
ButTyrOEt	6.0	0.015 0.014	0.0065 „	2.3	1.0
NvalTyrOEt	3.0	0.0070 „		2.3	
NleuTyrOEt	3.0	0.0054 „	0.0036 „	1.8	1.2
β-AlaTyrOEt	0.23	0.040 „	0.041 „	174	178
ValTyrOEt	0.37	0.016 0.014	0.0062 „	38	17
LeuTyrOEet	6.0	0.020 „	0.013 „	3.3	2.2
ε-NleuTyrOEt	0.045	0.0089 „		198	

1) $K_{enz.} = K_{obs.} - K_{no\ enz.}$

2) $K_{hyd.\ enz.} = K_{hyd.\ obs.} - K_{hyd.\ no\ enz.}$

3) $C = K_{enz.}/E$. E is the protein concentration in mg. N per ml. of assay solution.

4) $C_{hyd.} = K_{hyd.\ enz.}/E$

5) Substrate concentration., 0.05 *M*.

In a previous communication (*2*), it was shown that the hydrolytic rates by chymotrypsin of some amide substrates were increased markedly by the presence of large side chain groups in the N-terminal L-amino acid residue of aminoacyltyrosinamides. For example, leucyltyrosinamide is hydrolyzed at 25 times the rate for glycyltyrosinamide at pH 8.0. As may be seen from Table VI, the substitution of the glycyl residue of GlyTyrOEt by a larger L-amino acid residue leads to a marked diminution in the rate of disappearance of ester. For example, LeuTyrOEt disappears at 1/43 times the rate for GlyTyrOEt.

It is of interest to note that the appearance of the change in the rate of hydrolysis by cathepsin C of various aminoacyl-tyrosine or -phenylalanine amides is similar to that of various corresponding ester substrates (*9*, *14*).

SUMMARY

1. A number of aminoacyl-L-tyrosine ethyl ester hydrochlorides have been synthesized and tested as substrates for α-chymotrypsin.

2. The stability of aminoacyltyrosine esters, in which the aminoacyl substituents are glycyl, L-alanyl, L-α-aminobutyroyl, L-norvalyl, L-norleucyl, L-valyl, L-leucyl, β-alanyl and ε-norleucyl groups, was tested in the absence of the enzyme at pH 8.0.

3. The pH optimum of chymotrypsin activity for glycyltyrosine ethyl ester was found to be near 8.3.

4. The enzyme catalyzes at least two reactions: hydrolysis of the ester linkage to form the corresponding dipeptide and formation of the ninhydrin negative product. β-Alanyl and ε-norleucyl tyrosine esters, however, are simply hydrolyzed, yielding no ninhydrin negative products.

5. The rates of action of the enzyme was decreased markedly by the presence of large side chain groups in the N-terminal L-amino acid residues of the substrates.

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DIPHOSPHOPYRIDINE NUCLEOTIDE L-GULONIC ACID DEHYDROGENASE FROM GUINEA PIG LIVER

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The enzymatic mechanism of the formation of L-xylulose from D-glucuronic acid in mammalian tissues has been studied by many investigators and the participation of L-gulonic acid dehydrogenases specific to triphosphopyridine* and diphosphopyridine nucleotide has been established in the liver and kidney of guinea pigs (*1*), or other species of animals (*2–4*). The reduction of D-glucuronic acid with TPNH in the presence of TPN-L-gulonic dehydrogenase has been confirmed as the first step of L-xylulose formation (*1*, *3*).

$$\text{D-glucuronic acid} + \text{TPNH} + \text{H}^+ \rightleftarrows \text{L-gulonic acid} + \text{TPN}^+$$

In the previous paper from this laboratory, L-gulonic acid, the product of the above reaction, was shown to undergo second reaction by the DPN-L-gulonic dehydrogenase (*1*). When a crude preparation of the liver or kidney of guinea pigs was used, L-gulonic acid was converted to L-xylulose with simultaneous release of carbon dioxide in the presence of DPN.

$$\text{L-gulonic acid} + \text{DPN}^+ \longrightarrow \text{L-xylulose} + \text{CO}_2 + \text{DPNH} + \text{H}^+$$

However, ketohexonic acid, probably 3-keto-L-gulonic acid, was proposed as the reaction product of DPN-specific gulonic acid dehydrogenase by Lehninger and his coworkers (*2*, *3*), and this compound was suspected as the direct precursor of L-ascorbic acid or L-xylulose, in the following manner:

$$\text{L-gulonic acid} + \text{DPN}^+ \longrightarrow \text{3-keto-L-gulonic acid} + \text{DPNH} + \text{H}^+$$
$$\text{3-keto-L-gulonic acid} \longrightarrow \text{L-ascorbic acid} + \text{H}_2\text{O}$$
$$\text{3-keto-L-gulonic acid} \longrightarrow \text{L-xylulose} + \text{CO}_2$$

Thus, the DPN-specific enzyme was considered by these workers as an enzyme similar to malic enzyme, but the enzyme has not been purified as yet, and the nature of DPN-L-gulonic dehydrogenase has not been clarified completely.

The present report deals with the purification and properties of DPN-

* The following abbreviations will be used: Diphosphopyridine nucleotide, DPN; triphosphopyridine nucleotide, TPN; xylulose, Xu; dimethylaminoethylcellulose, DEAE- cellulose; ethylenediamine tetraacetate, EDTA; tris(hydroxymethylamino)methane, Tris; reduced glutathione, GSH; *p*-chloromercuribenzoate, PCMB.

L-gulonic dehydrogenase from guinea pig liver which is unable to synthesize L-ascorbic acid *in vivo* or *in vitro*.

EXPERIMENTAL

L-Gulonolactone and L-galactonolactone were synthesized from corresponding uronic acids by reduction with sodium borohydride (*5*). Free acids, L-gulonic acid and L-galactonic acid, were obtained by hydrolysis of the lactones with a stoichiometric amount of NaOH in hot water (55°). D-Mannoic acid was obtained by hydrolysis and subsequent reduction of alginic acid. L-Xu was synthesized by enzyme reaction starting from L-gulonic acid using L-gulonic acid dehydrogenase system, and purified by column chromatography using Dowex-1-borate. DEAE-cellulose was synthesized according to the method of Peterson and Sober (*6*). Toyo Roshi cellulose powder (× 100 mesh) was used as the starting material. DPN and TPN were purchased from Pabst Laboratories, and its purity was found to be 95 per cent or over.

The determination of Xu was made by cysteine-carbazole reaction (*7*) or by orcinol reaction (*8*). The standard for the color reactions of ketopentoses was determined, assuming that the color development obtained from ketopentoses in orcinol reaction is 60 per cent compared with aldopentose (*9*). The estimation of aldonic acids was carried out by periodate oxidation and subsequent chromotropic acid color reaction as in the determination of glycerol (*10*). The concentration of protein in the enzyme solutions was determined by ultraviolet absorption at 280 and 260 mμ according to Warburg and Christian (*11*).

The assay of enzyme reaction was carried out by the determination of change in optical density at 340 mμ due to the reduction of DPN using a Hitachi spectrophotometer equipped with cells having 1 cm. light pass. The standard reaction mixture contained 0.65 μM of DPN, 0.2 μM of $MnCl_2$, 10 μM of sodium L-gulonate, 50 μM of Tris buffer (pH 8.4), and 0.1 ml. of the enzyme solution (approximately 80 units). The total volume of the system was made to 3.0 ml. by the addition of isotonic KCl solution containing 1×10^{-3} *M* EDTA, or distilled water. The reaction was started by addition of the substrate and the determination was completed within 5 minutes. The reaction was carried out at room temperature or at 25°. The unit of the enzyme was defined as the change of optical density at 340 mμ in 5 minutes $\times 10^3$, and the specific activity of the enzyme was expressed as units per mg. of protein. The determination of optical rotation was carried out using a Zeiss micropolarimeter, furnished with 3 × 50 mm. tube.

RESULTS

Purification of the Enzyme—Young guinea pigs each weighing about 300–350 g. were sacrificed by decapitation under ether anaesthesia, and the livers from 10 guinea pigs were pooled in cold isotonic KCl solution containing 1×10^{-3} *M* EDTA. After blotting, the tissue was weighed and homogenized with 2.5 volume of the same KCl solution using a Potter-Elvehjem glass homogenizer. The homogenate, after adjusting to pH 7.5, was immediately subjected to centrifugation at $100{,}000 \times g$ during 30 minutes at 0°. The resulting precipitate was discarded and a clear supernatant was pooled (about 230 ml.). All the subsequent steps were carried out at 0 to 4°.

Ammonium Sulfate Fractionation I—To the above supernatant, neutralized

saturated ammonium sulfate solution was added slowly to the final concentration of 38.5 per cent*. The mixture was immediately centrifuged in the cold at 4,000 r.p.m. during 15 minutes to remove the precipitate. To the supernatant, ammonium sulfate solution was further added to the concentration of 53.5 per cent. The solution was again centrifuged and the supernatant was discarded. The precipitate was dissolved in 0.04 *M* Tris buffer (pH 7.5) containing 1×10^{-3} *M* EDTA and the enzyme solution was dialyzed overnight against about 2 liters of the same buffer.

Negative Adsorption with DEAE-cellulose—After the small amount of precipitate in the dialyzed solution was centrifuged off, the supernatant was diluted with an equal volume of 0.08 *M* Tris buffer (pH 7.5). Total volume of the enzyme solution became about 160 ml. To this solution, one-third volume of DEAE-cellulose-Cl, previously buffered with 0.06 *M* Tris buffer (pH 7.5) containing 1×10^{-3} *M* EDTA, was added. The mixture was placed in an ice bath and stood for 60 minutes with occasionl stirring. DEAE-cellulose was removed by centrifugation and washed with 0.04 *M* Tris buffer.

Ammonium Sulfate Fractionation II—To the combined supernatant, saturated ammonium sulfate solution was added to a concentration of 45 per cent during 40 minutes. The precipitate was removed by centrifugation and ammonium sulfate was further added to the supernatant until its concentration reached 60 per cent. The precipitate was collected by centrifugation and dissolved in minimal volume of 0.1 *M* phosphate buffer (pH 7.4) containing 1×10^{-3} *M* EDTA. This preparation was fairly stable and could be

TABLE I

Purification of the Enzyme

	Specific activity (*units/mg.*)	Recovery of activity for each step (*per cent*)	Total protein (*mg.*)	Volume of the fraction (*ml.*)
Original supernatant	<2	—	—	220
Ammonium sulfate-I	10.2	90	1950	64
DEAE-cellulose	—	76	—	—
Ammonium sulfate-II	29.5	91	435	14

stored in a refrigerator for several weeks without any noticeable loss of activity, although cruder preparations lost the activity rapidly during storage. Storage in a deep freeze was avoided because the loss of the activity occurred by repeated freezing and subsequent thawing. The yield and specific activity for the each step are given in Table I.

* The concentration of ammonium sulfate solution is expressed by volume per volume per cent in the following description of the purification.

The preparation of DPN-L-gulonic dehydrogenase obtained above, still contained TPN-L-gulonic dehydrogenase, sorbitol dehydrogenase, and lactic dehydrogenase but not L-gulonolactonase. Further purification was attempted by column chromatography using DEAE-cellulose; after the ammonium sulfate fractionation I and dialysis against Tris buffer, chromatographic separation of the enzyme was undertaken with Tris buffer as a developing agent.

As can be seen from Table II, the DPN-enzyme was separated from the TPN-enzyme by this procedure. The DPN-enzyme fractions did not contain sorbitol dehydrogenase and a higher specific activity was attained. However, the recovery of enzyme activity was low and the preparation obtained by this procedure rapidly lost the activity during storage. Treatment with organic solvents, including acetone dried powder procedure, could not be used for the preparation of this enzyme due to its instability to these agents. During the course of this stuby it was found that the activity of the enzyme in the original extract occasionally showed a marked fluctuation and instability as reported by Hollman (*12*), contrary to the almost constant activity shown by TPN-L-gulonic dehydrogenase of the same level of purity. The reason for this phenomenon was not elucidated.

TABLE II

Chromatography of DPN-Enzyme

The enzyme solution was dialysed over night against 0.04 *M* Tris-HCl buffer pH 7.5 containing 1×10^{-3} *M* EDTA. The resultant precipitate was centrifuged off and the supernatant was diluted with equal volume of water. DEAE-cellulose-Cl was prepared by the addition of sufficient volume of 0.1 *N* HCl and bufferized with 0.02 *M* Tris buffer pH 7.5 containing 5×10^{-5} *M* EDTA. The resin was filled in a column of the size 25×40 mm. and the avove enzyme solution was poured into column at a flow rate of 8–12 drops per minute. All the procedures were conducted at about 2°.

	Protein concentration (*mg./ml.*)	Specific activity		Concentration of Tris buffer (*M*)
		DPN-enzyme (*units/mg.*)	TPN-enzyme (*units/mg.*)	
Original enzyme, before dilution	21.2	5.5	3.3	0.04
Passed-through solution	0.96	12.2	0.0	0.02
Tube number*				
5	0	0.0	0.0	0.08
7	0.87	55	0.0	0.08
9	2.20	27.5	3.5	0.08
12	0.82	34.0	61	0.08
20	0.42	0.0	100	0.08

* Volume of each fraction was 10 ml.

Properties of the Enzyme—Optimum pH for the enzyme activity was at about 8.4 as determined with Tris buffer. Contrary to the rapid decrease of activity in a more acidic side of pH, only slight decrease was observed in alkaline side. Phosphate buffer gave similar results as Tris buffer.

Metal Requirement—Manganese ions exhibited considerable activation at the concentration of 1×10^{-5} *M* in the absence of EDTA and KCl, whereas 10 fold higher concentration was required in the presence of 3.3×10^{-5} *M* EDTA, and in this case, activation was observed to a much smaller extent. All the preparations obtained were active even in the absence of manganese

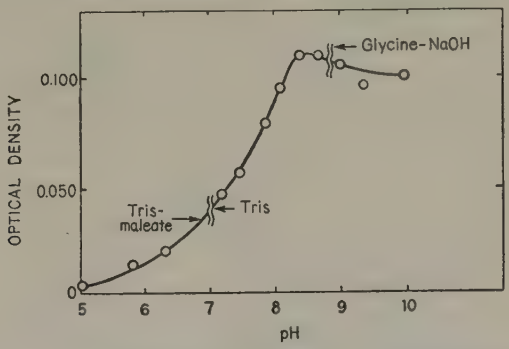

FIG. 1. Optimal pH of DPN-gulonic dehydrogenase.
pH 5.1～ 6.25 0.05 *M* Tris-maleate buffer.
pH 7.2～ 8.65 0.05 *M* Tris buffer.
pH 9.0～10.0 0.05 *M* Glycine buffer.

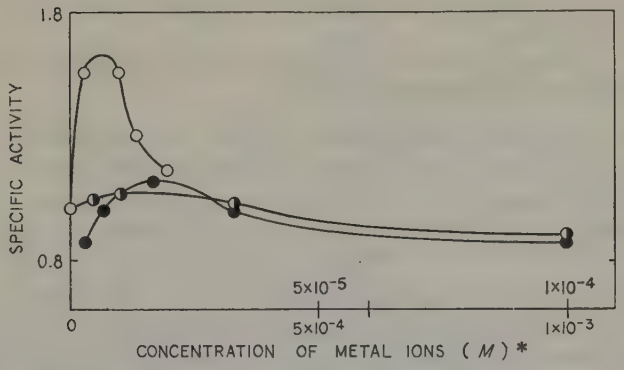

FIG. 2. Effect of metal ions on DPN-gulonic dehydrogenase.**
* Scales: without EDTA (above) and with EDTA (below).
** ○ Mn^{2+}
◑ Mn^{2+}, EDTA 3.3×10^{-5} *M*
● Mg^{2+}, EDTA 3.3×10^{-5} *M*

ions. Magnesium ions were less effective than Mn^{++}. All other divalent cations tested, including Sr^{++}, Ca^{++}, Co^{++} and Fe^{++}, had no effect on the enzyme activity.

Substrate Specificity—The enzyme was active on D-mannoic acid to 33 per cent relative to L-gulonic acid under the same conditions. L-Galactonic acid, D-gluconic acid, 2-keto-L-gulonic acid, L-gulonolactone, D-glucuronic acid diethylmercaptal neither served as substrate, nor inhibited the enzyme action competitively. These findings indicate high degree of substrate specificity of this enzyme. The Michaelis constant of the enzyme was determined according to Lineweaver and Burk (*13*). K_m for L-gulonic acid was estimated as 4.3×10^{-4} mole per liter. As for the DPN, a linear relationship was not found between the reciprocal of DPN concentration and initial velocity of the reaction.

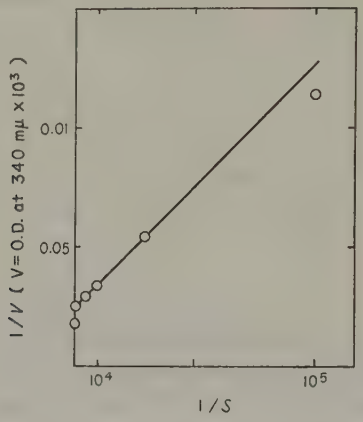

FIG. 3. Rate of the reaction as a function of L-gulonic acid concentration.

The assay systems were as described in the method and the experiments were carried out at 25°.

Cofactors—Thiamine pyrophosphate, adenosine triphosphate and uridine triphosphate, which had been reported as effective cofactors in glucuronolactone decarboxylation in the rat liver supernatant system (*14*), did not show any effect upon this enzyme activity. Adenosine-5′-P was also ineffective.

Inhibitors—Heavy metal ions, such as Cd^{++} or Zn^{++}, strongly inhibited the enzyme activity at concentrations of 10^{-4} *M* or less. *p*-Chloromercuribenzoate completely inhibited the activity at the concentration of 2×10^{-5} *M*, when it was added to the test system after the addition of Mn^{++} and DPN. The inhibition was reversed by the addition of 10^{-3} *M* of GSH following PCMB. GSH alone activated the enzyme to the extent of some 40 per cent in the case of stored enzyme. The thiol nature of the enzyme can be considered from these findings. High concentrations of EDTA also inhibited the enzyme activity. Cyanide ions of concentration of 1×10^{-2} *M* showed 17 per cent inhibition at the optimum pH for the enzyme reaction. At pH 7.4, 0.03 *M* potassium cyanide strongly inhibited the enzyme activity. Because

CN-DPN complex is formed at an appreciable velosity under such a high concentration of cyanide ions, it is not clear whether this inhibition was caused by CN-DPN complex or by cyanide ions.

Enklewitz (*15*) reported that, when antipyrine was administered to the pentosuric human, the excretion into the urine of L-xylulose originating from D-glucuronic acid was considerably increased. This effect of antipyrine has been considered as due to the action of 4-aminoantipyrine, which was metabolically produced from antipyrine, on the decarboxylation of β-keto acid derived from D-glucuronic acid. However, 4-aminoantipyrine did not have any appreciable effect on the purified enzyme except that at a higher concentration of 10^{-2} *M* or more, both the dehydrogenation and decarboxylation reactions were slightly inhibited. Formation of a small amount of ketohexonic acid by the purified enzyme from pig kidney was supposed by

Table III

Effect of Inhibitors

Compounds	Concentrations added (*M*)	Inhibition or activation (*per cent*)
PCMB	1.3×10^{-7}	0
„	3.3×10^{-6}	− 44
„	2.0×10^{-5}	−100
PCMB +GSH	2.0×10^{-5} + 3.3×10^{-3}	− 5
Cd^{++}	1×10^{-4}	−100
Zn^{++}	2×10^{-4}	− 89
CN^- (at pH 7.5)	4×10^{-3}	− 19[1]
„	1×10^{-2}	− 42
„	3×10^{-2}	− 83
4-Aminoantipyrine (Dehydrogenation reaction)	3.3×10^{-2}	− 8
(Coupled reaction)	1×10^{-2}	− 15[2]
GSH	3.3×10^{-3}	+ 47

The conditions of tests were the same as described.

1) Absorption at 340 mμ due to CN-DPN complex formation was substracted from the reading of 340 mμ.

2) Conditions for coupled reaction was similar to those described under Fig. 4.

Ashwell (*16*) from the finding that a minute amount of L-Xu was formed nonenzymatically with simultaneous release of Co_2 when 4-aminoantipyrine was added to the system after stoppage of the enzymatic reaction. In the present study, using guinea pig liver enzyme, extra carbon dioxide production was not detected on the addition of 4-aminoantipyrine at pH 5.2 at the

end of the coupled reaction with lactic dehydrogenase and pyruvic acid.

Stoichiometry of the Reaction—Precise stoichiometry of the reaction was found by the chemical determination of L-Xu formed compared with the DPN reduced. However, such a chemical determination could not exclude the possibility that labile keto acid might have been determined as Xu. The absorption spectrum of the cysteine-carbazole reaction product was identical

TABLE IV

Stoichiametry of the Reaction

Time min.	DPNH formed μM/ml.	L-xylulose formed μM/ml.
11	0.031	0.031
22	0.051	0.051
40	0.068	0.074
0	0.00	0.00

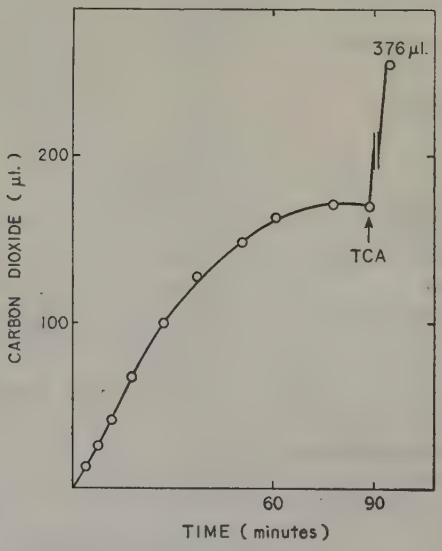

FIG. 4. Decarboxylation reaction.

The test system contained 20 μM of Na-gulonate in the side arm-I; 20 μM of Na-pyruvate, 20 μM of Tris buffer (pH 7.5), 1.4 μM of DPN, 0.2 μM of Mn^{++} as a chloride, 0.2 ml. of lactic dehydrogenase prepared by the method of Kornberg and Pricer (*17*), and 1.0 ml. of gulonic dehydrogenase, in the main chamber; and 0.4 ml. of trichloroacetic acid in the side arm-II. The total volume of the system was made up to 3.0 ml. The gasphase was air; temperature 37°. The reaction was started by tipping L-gulonate from the side arm-I and stopped by the addition of trichloroacetic acid from the side arm-II. Controls in which L-gulonate was omitted showed no CO_2 evolution before or after trichloroacetic acid addition.

with that of ketopentoses.

Decarboxylation Reaction—The purified enzyme catalysed the reaction of L-gulonic acid coupled with lactic dehydrogenase and pyruvic acid, as previously shown with the crude enzyme (*1*). Decarboxylation of L-gulonic acid started immediately after the addition of DPN or the substrate and proceeded almost to completion.

The Reaction Product—Formation of Xu was previously demonstrated by paper chromatography (*1*). In view of the possibility of the formation of any acidic product including 3-keto-L-gulonic acid, the separation of the reaction product was attempted by column chromatography using Dowex-

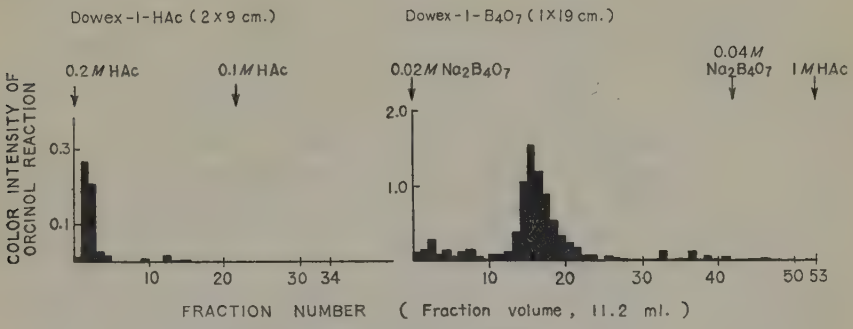

FIG. 5. Chromatography of the reaction product.

Incubation mixture for the large scale experiments contained 400 μM of Na-gulonate, 400 μM of Na-pyruvate, 12 ml. of gulonic dehydrogenase preparation at the purification stage of ammonium sulfate-I, 1.0 ml. of lactic dehydrogenase (*17*), 2 μM of $MnCl_2$, 7.0 *M* of DPN, 400 μM of Tris buffer (pH 7.4), and 960 μM of nicotinamide in a total volume of 25.1 ml. Gas phase was air; temperature 37°. The mixture was incubated for 90 minutes and the reaction was stopped by immersion of the vessel into a boiling water bath for 5 minutes. After cooling in an ice bath, denatured protein was centrifuged off and the precipitate was washed with a small volume of water. The supernatant and washing were combined and diluted to 300 ml. with water. The solution was passed through a column of Dowex-1-acetate and the effluent from the column was received in a vessel containing Amberlite IR-120-H so that pH of the solution could be maintained around neutral. Decationized solution was further deionized by Dowex-1-OH and concentrated under reduced pressure to about one-tenth of the original volume. Solid sodium tetraborate was added to a concentration of 0.02 *M*. The solution was passed through a column of Dowex-1-borate and developed according to the method of Lampen (*18*). Pooled orcinol positive fraction was deionized with Amberlite-IR-120-H and dehydrated under reduced pressure. Boric acid was removed as methanol borate, and the Xu thus obtained was identified by the orcinol and cysteine-carbazole reactions as described previously (*1*). L-Xu used for the experiments was obtained according to this procedure. Value of optical rotation was $[\alpha]_D^{22}+37°$ (c=1.54).

1-borate. The result is presented in Fig. 5. From the Dowex-1-acetate column, only the added DPN was recovered as orcinol-positive substance, and other suspected intermediate, which might be orcinol positive by itself

or after hydrolysis, was not obtained. From the Dowex-1-borate column, Xu was obtained as an orcinol-positive peak, and identified as L-Xu by color reactions and determination of its optical rotation (*1*).

Reversibility of the Reaction—If DPN-gulonic dehydrogenase is similar to malic enzyme or 6-phosphogluconic dehydrogenase in properties, demonstration of reversibility of the reaction starting from L-Xu, DPNH, and CO_2 would be expected. Slow oxidation of DPNH in the presence of L-Xu was observed with the purified enzyme. However, this oxidation of DPNH was not dependent on carbon dioxide. L-Xu reductase of Hollman and Touster (*19*) was reported as particle-bound and the nature of this

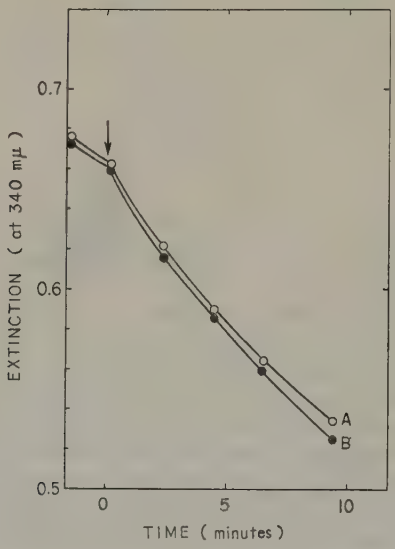

FIG. 6. DPNH oxidation in the presence of L-Xu.

Each tube contained 3.7 μM of L-Xu, 0.395 μM of DPNH of prepared according to Colowick (*22*). 100 μM of Tris buffer (pH 7.4), 0.2 μM of $MnCl_2$, 0.5 ml. of L-gulonic dehydrogenase. 600 μM bicarbonate, gassed with CO_2 immediately before use, was added in the experiment A, and this was replaced by water in the experiment B. L-Xu was added to the system at the point indicated by an arrow.

xylulose reduction will have to be studied further. This situation is somewhat similar to the reaction catalyzed by DPN-isocitric dehydrogenase of mitochondria which was reported also as being irreversible (*20*). DPN-dependent incorporation of $C^{14}O_2$ into the carboxyl carbon of gulonic acid was reported by Bublitz using a rat liver enzyme system (*21*). All attempts to demonstrate such incorporation of $C^{14}O_2$ in the presence of the purified enzyme and DPN have so far been unsuccessful.

DISCUSSION

As a result of the experiments described above, the DPN-L-gulonic dehydrogenase from guinea pig liver may be considered as the double headed enzyme similar to isocitric dehydrogenase and malic enzyme, functioning both in dehydrogenation and β-decarboxylation, though the difficulty of attaining higher degree of purification makes this statement inconclusive. The failure to separate dehydrogenation from decarboxylation, activation of the enzyme by manganese ions, the ineffectiveness of keto-fixatives such as cyanide for accelerating the enzyme reaction in the absence of Mn^{++}—all these findings are consistent with statement. However, direct evidence for the participation of 3-keto-L-gulonic acid as intermediate could not be demonstrated. In the hog liver system, Ashwell (*16*) reported the formation of a small amount of keto-hexonic acid, which was probably 3-keto-L-gulonic acid. In the case of guinea pig liver system, no positive evidence for the accumulation of such keto-hexonic acid was obtained. More suitable methods of analysis, such as the enzymatic determination of L-Xu (*23*) or the use of labeled gulonic acid (*16*), would give a definitive answer to this problem. Accumulation of keto acid in this enzyme reaction observed by Ashwell might also be considered as resulting from dissociation of the reaction intermediate from a single enzyme protein rather than the reaction product of first dehydrogenase reaction in a series of two consecutive reactions catalyzed by two different enzyme proteins. In the case of isocitric dehydrogenase or malic enzyme, the reaction intermediate dose not appear in the surrounding media at all and this is interpreted as being due to a very small dissociation constant of this intermediate. There remains, however, a possibility that another DPN-L-gulonic dehydrogenase, as distinct from the enzyme studied here and producing 3-keto-L-gulonic acid as the reaction product, is responsible for the formation of ascorbic acid.

The failure to demonstrate optically the reverse reaction and the absence of DPN-dependent $C^{14}O_2$ incorporation into gulonic acid suggests the irreversible nature of this enzyme reaction. In this connection, it is of interest to recall that the reactions catalyzed by DPN-isocitric dehydrogenase of yeast (*24*) or mammalian heart mitochondria (*20*) are also irreversible. Furthermore, cyanide ions inhibited both enzymes at the concentration of 0.01 *M* or higher. Thus the DPN-L-gulonic dehydrogenase might be regarded as the enzyme falling into the same category as DPN-isocitric dehydrogenase, although the requirement for adenylic acid was not demonstrated in the gulonic dehydrogenase.

Almost complete inhibition of the enzyme activity by higher concentration of cyanide ions is interesting in contrast with the acceleration of the formation of L-ascorbic acid from D-glucuronic acid by cyanide ions in a goat liver system reported by Guha and coworkers (*25*). From the results of the present study it might be supposed that the effect of CN on the formation of ascorbic acid is due to the inhibition of DPN-L-gulonic dehy-

drogenase which results in blocking one of the metabolic pathways of L-gulonic acid at the bifurcation point in the course of D-glucuronic acid metabolism, though the other pathway leading to the formation of L-ascorbic acid is not elucidated.

DPN-L-gulonic dehydrogenase, coupled with the TPN-L-gulonic dehydrogenase may constitute the main pathway of D-glucuronic acid metabolism in the guinea pig liver. In the tissues synthesizing ascorbic acid, another system which acts upon L-gulonic acid, would be operative (*26*).

SUMMARY

DPN-L-Gulonic dehydrogenase was purified from guinea pig liver and its properties were described.

1. The enzyme was purified about 20 fold using ammonium sulfate fractionation and negative adsorption with DEAE-cellulose.
2. The purified enzyme was separated from TPN-L-gulonic dehydrogenase, sorbitol dehydrogenase, and gulonolactonase, but dehydrogenation reaction could not be separated from decarboxylation reaction.
3. L-Xu was identified as the reaction product.
4. The reverse reaction was not demonstrated.
5. Some properties of the enzyme were discussed compared to other β-dehydrogenation-decarboxylation enzymes.

The author wishes to thank Dr. N. Shimazono for his continuous interest and advice during the course of this investigation, and is grateful to Miss K. Noguchi for her skillful technical assistance. The author is also indebated to Drs. H. Masamune of Tohoku University, Y. Takagi of Osaka University, T. Nakajima of Pharmaceutical Department of Tokyo University, S. Nasuno of Institute of Applied Microbiology of Tokyo University for their kind supply of some of the materials used in this work. This work was supported in part by a grant for scientific research from the Ministry of Education.

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STUDIES ON GLUCURONOLACTONASE AND GULONOLACTONASE

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It has been established by many authors (*1–4*) that D-glucurono-γ-lactone and L-gulono-γ-lactone, as well as D-glucuronic acid and L-gulonic acid, are precursors of L-ascorbic acid in animal tissues. Touster and others (*5–6*) found L-xylulose excreted in the urine of essential pentosuric humans and in the urine of a guinea pig fed with D-glucuronolactone. Recently, Ishikawa *et al.* (*7*) and Lehninger (*3*, *4*, *8*) have proved that the liver and kidneys of a guinea pig contained DPN- and TPN-specific dehydrogenases of L-gulonic acid, and that the reaction products were xylulose in the DPN system and glucuronic acid in the TPN system. Ishikawa (*9*) further observed that these dehydrogenases possessed activity on glucuronic acid or gulonic acid but not on glucuronolactone or gulonolactone. These reports indicated that there exists a route from D-glucuronolactone and L-gulonolactone to their corresponding acids.

In the past, the presence of an enzyme activity hydrolyzing these two lactones was reported by Lehninger (*3*) and Eisenberg (*10*) in the homogenate or slices of rat liver. However, no report on the purification and detailed properties of these glucurono- and gulonolactonase has been published.

In the previous report (*11*) on the distribution of lactonase in various animals, Yamada *et al.* showed that gulonolactonase seemed to play a significant role in the biosynthesis of L-ascorbic acid.

The present paper describes the preparation and purification of the enzyme, together with an account of some of its properties. In this study, the distribution of lactonase activity in cell fractions showed the presence of two evidently different lactonases, tentatively named Lactonase-I and -II for soluble and microsomal fractions, respectively.

EXPERIMENTAL

Chemicals

L-Gulono-γ-lactone was prepared by the reduction of D-glucuronate with sodium boroҳydride according to the method of Wolfrom and Anno (*12*), D-galactono-γ-lactone from galactose by oxidation with bromine (*13–14*), D-gluconolactone from gluconic acid (*15*), and

L-glucono-γ-lactone and D-idono-γ-lactone from D-xylose by the convenient modified method of the Kiliani synthesis as described by Hudson (*16*). Ethyl ester of D-glucuronic acid was donated by Dr. T. Nakazima, Department of Pharmacology, University of Tokyo. L-Galactono-γ-, L-glucono-δ-, D-mannono-γ-, and D-manurono-γ-lactone were generously donated by Dr. H. S. Isbell of the National Bureau of Standards. D-Glucurono-γ-lactone was obtained from the Chugai Seiyaku Co., Ltd.

DEAE-cellulose was synthesized from 2-chlorotriethylamine hydrochloride and cellulose (100-mesh, Toyo Roshi Co., Ltd.) by the method of Peterson and Sober (*17*), using 3 times the volume of 2-chlorotriethylamine hydrochloride as that of the original method.

Purification of the Enzyme (Lactonase-I)

Step 1. Preparation of the Bovine Liver Acetone Powder—Fresh bovine liver was dissected with scissors into thick pieces and was blended with 5 volumes of acetone, cooled to $-20°$ with dry-ice, for about 3 minutes in a Waring blendor. After filtration through a Buchner funnel with suction, the semi-dry filter cake was retreated with acetone and again filtered. This treatment and filtration were repeated once more. The filter cake was ground in a mortar to dry quickly. It was then stored in a desiccator at room temperature. Under these conditions the powder was stable for 6 months.

Step 2. Extraction—10 g. of the acetone powder was extracted with 8 volumes of ice-cold redistilled water during 30 minutes with occasional stirring. The clear supernatant was obtained by centrifugation at 3,000 r.p.m. for 5 minutes.

Step 3. Heat Treatment—The extract in a 500 ml. flask was placed in a 73° bath and stirred continuously. The temperature of the extract rose to 62~63° in about 4~5 minutes and was maintained at 63° for 3 minutes, then placed in an ice bath. Denaturated proteins were contrifuged off.

Step 4. Fractionation with Ammonium Sulfate—To 53 ml of the solution 21 g. of solid ammonium sulfate were added under continuous stirring to give 0.52 saturation at 0°. After standing for 30 minutes, the precipitate was removed by centrifugation (4,000×*g*, 10 minutes) in a refrigerated room. The supernatant fluid was brought to 0.67 saturation and, after centrifugation, the 0.52~0.67 fraction was dissolved in abont 20 ml. of 0.01 *M* Tris-buffer (pH 7.35), and dialyzed in the cold against 1 liter of redistilled water during 2 days, with several changes of water.

Step 5. Fractionation with Acetone at pH 6.8—The dialyzed solution was adjusted to pH 6.8 with Tris or HCl, and Cl^- concentration was brought to 0.01 *M* with addition of water. Acetone, cooled to $-10°$, was added slowly with constant stirring to achieve the final concentration of 40 per cent by volume. Final temperature was $-2\sim\rightarrow 5°$. After standing for 10 minutes, the precipitate was removed by centrifugation (4,000×*g*, 10 minutes) at same temperature. Acetone was then added to the supernatant to a final concentration of 55 per cent by volume. The precipitate was collected by centrifugation and dissolved in 70 ml. of 0.01 *M* Tris buffer* of pH 7.75.

Step 6. Column Chromatography—Prior to use, 200 ml. of 1 *N* NaOH was added to 5 g of DEAE-cellulose. After a settling period of 1 hour, thes upernatant suspension was decanted and the precipitate was filtered through a Buchner funnel with suction. Then 200 ml. of 1 *N* HCl was added and the mixture was filtered after 10 minutes. The resin was washed

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The following abbreviations are used in this prper: DPN: diphosphopyridine nucleotide, TPN: triphosphopyridine nucleotide, Tris: tris (hydroxymethyl) aminomethane, DEAE-cellulose: diethylaminoethyl-cellulose, PCMB: *p*-chloromercuribenzoate.

* in this paper, mole-concentration of Tris buffer is shown by its Cl^- concenration.

with 0.01 *M* Tris buffer of pH 7.75 until its pH reached 7.75. The resin was bufferized overnight with the same buffer.

The column, using 4 g. of the bufferized DEAE-cellulose, was prepared in a chromatographic tube with a diameter of about 5 cm., plugged with glass fiber. The column was washed with 100 ml. of the same buffer. Then, 70 ml. of the enzyme solution dissolved in 0.01 *M* Tris buffer of pH 7.75 was poured into the column, with pumping by means of a suitably controlled constant air pressure, at a flow rate of 10–15 ml. per 10 minutes. The column was then washed with 100 ml. of the same buffer and eluted with 0.01 *M* Tris buffer of pH 7.35 containing 0.02 *M* NaCl. The flow rate was 10–15 ml. per 10 minutes at a constant room temperature of 5° and the effluent was collected in fractions of 5~10 ml. Analysis of effluent fractions is shown in Fig. 1. This effluent fraction has the best storage properties, being fairy stable for 3 weeks at around 0°.

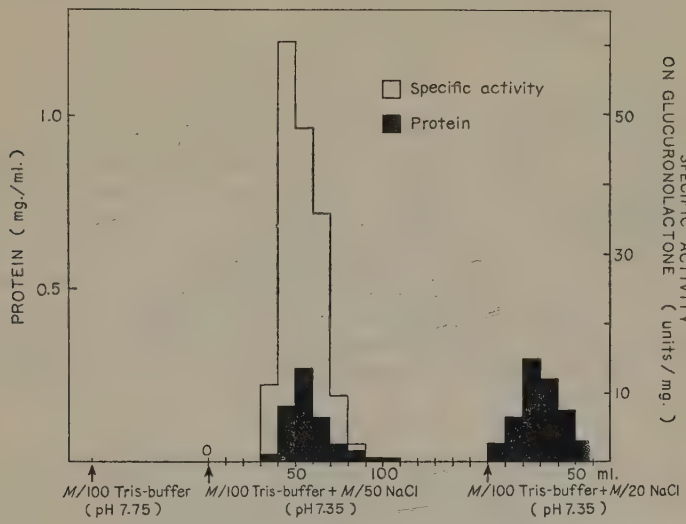

Fig. 1. Chromatographic purification of Lactonase-I on DEAE-cellulose column.

Summary of purification procedure is shown in Table I. Finally, the enzyme was purified 21-fold from acetone powder.

Preparation of the Cell Component

Fractions of cell component, namely mitochondria, microsome, and soluble fraction, were prepared by the method of Hogeboom (*18*) with some modifications. In this procedure, two-layer method of 0.25 *M* and 0.35 *M* sucrose was applied for the isolation of mitochondrial fraction, although Hogeboom used this method to isolate a nuclear fraction.

Assay Method

Protein—The protein content of the enzyme solution was calculated by estimating optical density of the solution at both 280 and 260 mμ, according to the method of Warburg and Christian (*19*).

TABLE I

Summary of Purification Procedure

	Specific Activity on Glucuronolactone (*units/mg.*)	Recovery (*%*)	Activity Ratio Gul-L/Gl-L	Total Protein (*mg.*)
Acetone powder (Water extract 63 ml.)	2.8	100	3.4	790
Heat treatment (54 ml.)	7.6	86	3.3	246
Ammonium sulfate fractionation, dialysis (39 ml.)	12.5	43	3.3	75
Acetone fractionation (20 ml.)	—	17	3.4	—
DEAE-cellulose column treatment	60.5	9	3.4	4.3

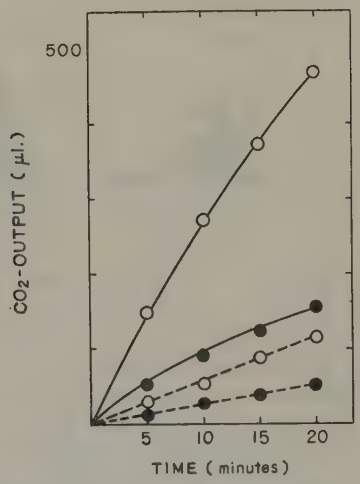

FIG. 2. Manometric assay of enzyme activity

Reaction mixture contained 0.4 ml. of 0.1 *M* sodium bicarbonate, 0.1 ml. of 0.1 *M* magnesium sulfate, 0.3 ml. of 0.001 *M* glutathione, and 0.2 ml. of enzyme solution; side arm, 0.3 ml. of 0.1 *M* substrate; gas phase 93 per cent of N_2 and 7 per cent of CO_2; temperature, 37°; pH, 7.2; substrate was tipped in after incubation for 10 minutes. —○— gulonolactone, ---○--- gulonolactone non-enzymatic, —●— glucuronolactone, ---●--- glucuronolactone non-enzymatic

Manometric Method—The enzyme was assayed manometrically by the measurement of CO_2 evolution, in a bicarbonate-carbon dioxide buffered solution, since the acid produced by hydrolysis converted bicarbonate to CO_2. Conditions are shown in Fig. 2.

Colorimetric Method—Residual lactone was measured by an adaptation of the hydroxamic acid procedure of Eisenberg (*10*). This method was applied in 0.1 *M* phosphate buffer for the experiments of back reaction, and of the metal which precipitated in bicarbonate buffer, and in the case that it was important to know exactly the pH of the reaction mixture.

Definition of Specific Activity—Specific activity was expressed as the micromole of CO_2 output during 10 minutes of enzyme action per milligram of enzyme protein.

Unit of Enzyme Activity—Unit of enzyme activity was defined as the activity that produced 1 micromole of CO_2 during 10 minutes.

RESULTS

Reaction Products

Glucuronolactone and gulonolactone were hydrolyzed to glucuronic acid and gulonic acid by this enzyme. The glucuronic acid and gulonic acid were identified by paperchromatography, as follows: The reaction mixture was deproteinized with addition of 0.3 ml of 60 per cent perchloric acid and filtered. The filtrate was neutralized with KOH, the percipitated potassium-perchlorate was removed by filtration, and then this filtrate was concentrated by lyophilization. The residue was dissolved in a few drops of water and spotted on Toyo Roshi No. 53 filter paper. In Table II are listed the R_f values in various solvents. The spots were developed by the follwing two

TABLE II

Paper Chromatography of Reaction Product[1]

	Benzylalcohol acetic acid water 3:3:1	Butylalcohol acetic acid water 4:1:2[2]	Butylalcohol ethanol water 4:1:1	Hydroxamic reagent	Periodate starch reagent
	R_f	R_f	R_f		
D-Glucuronic acid	0.41	0.24		3)	White
D-Glucuronolactone	0.64	0.47		Red brown	White
L-Gulonic acid	0.51	0.37	0.09	3)	White
L-Gulonolactone	0.58	0.43	0.28	Red brown	White
Reaction product					
Substrate: D-Glucuronolactone	0.41	0.25		3)	White
Substrate: L-Gulonolactone	0.52	0.37	0.09	3)	White

1) Ascending method; front movement, about 300 mm.
2) As a control, glucose was tested, its R_f was 0.41.
3) Yellow, but immediately disappeared.

methods: Buffered, neutral hydroxylamine reagent and a solution of $FeCl_3$ described by Eisenberg (*10*), and aqueous KIO_4 and borate-soluble starch reagent according to Metzenberg (*20*).

Properties of Lactonase-I

Effect of pH—The optimum pH for the glucuronolactone as well as gulonolactone was about 6.8–7.0 at 37°, with phosphate buffer. At pH 6.0 the enzymatic hydrolysis of glucuronolactone or gulonolactone was disappeared completely. At above pH 7.4, enzymatic hydrolysis can not be discussed exactly, since, non-enzymatic hydrolysis increased markedly in such a pH range.

Activation and Inhibition—0.01 *M* of Mg^{++} and 10^{-4} *M* of Mn^{++} ions caused a 130 per cent and 60 per cent stimulation of activity, respectively. There was no such effect in Co^{++}, Fe^{++}, or Zn^{++} ions (Fig. 3).

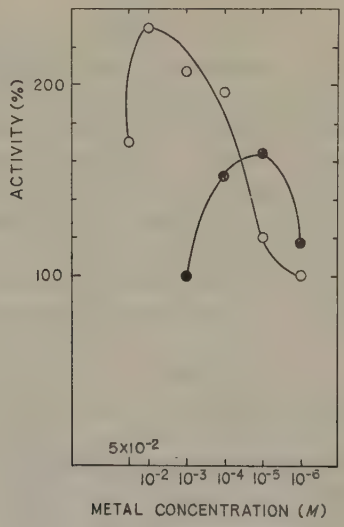

Fig. 3. Metal requirement
—○— Mg^{++}, —●— Mn^{++}

The enzyme was inhibited by heavy metals such as Hg^{++}, Ag^{+}, and Cu^{++}, in very low concentrations. Inhibition was also caused by PCMB, monoiodate, namely SH-blocking reagents. Reversal of this inhibition occurred if glutathione or cysteine was added within a short time after the addition of heavy metal ions or SH-blocking reagents. However, the reversal with glutathione was dependent on the presence of Mg^{++} ion (Table III).

Back Reaction—The back reaction was studied at pH 6.0 and 6.6 of 0.1 *M* phosphate buffer by the addition of 0.2 *M* neutral hydroxylamine (neutralized with NaOH) as trapping agent. After 90 minutes' incubation, a small amount of hydroxamate was found. When 200 μM of sodium glucuronate was used as the substrate, lactone formation was 8 μM at pH 6.0 and 6 μM at pH 6.6, but with sodium gulonate, it was less than 1 μM. In this experiment, HCl,

Table III

Inhibition by Heavy Metal Ions and Other SH-Blocking Reagents, and the Reversal of Glutathione and Cysteine

		Cu^{++}	Ag^{+}	Hg^{++}	PCMB	Monoiodo-acetic acid
Concentration (*M*)		8×10^{-6}	1×10^{-5}	1×10^{-6}	7.5×10^{-6}	1×10^{-2}
Inhibition (%)		100	87	71	100	87
Glutathione Conc. (*M*)		—	1×10^{-4}	—	1×10^{-5}	1×10^{-4}
Reversal (%)	Mg (+)	—	100	—	142	107
	Mg (−)	—	22	—	0	0
Cysteine Conc. (*M*)		1×10^{-3}	—	1×10^{-3}	1×10^{-3}	—
Reversal (%)	Mg (+)	17	—	50	44	—
	Mg (−)	17	—	50	24	—

Table IV

Substrate Specificity of Lactonase-I

Substrate -lactone[1]	Activity[2] (%)
L-Gulono-	100
D-Gulono-	278
L-Galactono-	139
D-Galactono-	160
L-Glucono-	20
D-Glucono-	190
D-Mannono-	31
D-Manurono	75
D-Glucurono-	31
D-Idono-	0
D-Glucono-δ-	29
D-Glucuronic acid ethyl ester	0
Triacetyn	0
Glycerophosphate	0
Acetylcholine	0

1) Lactones were all γ-lactone, except D-glucono-δ-lactone.
2) Conditions tested were the same as in Fig. 2, and the activities are expressed as per cent to the activity on L-gulonolactone.

trichloroacetic acid, and solution of $FeCl_3$ were added to 2 ml of reaction mixture according to Eisenberg's method (*10*).

Substrate specificity—In order to examine the specificity for substrate, various lactones were investigated. The results are shown in Table IV. Lactonase-I had a broad specificity and L-gulonolactone was hydrolyzed about 3 times as rapidly as L-glucuronolactone. It seems that D-form of lactone was hydrolyzed more rapidly than its L-form. Esterase activity was not present in Lactonase-I.

Substrate Affinity Constant—The Michaelis constant, calculated by the method of Lineweaver and Burk (*21*), was found to be 2.33×10^{-3} mole per liter for glucuronolactone and 1.43×10^{-3} mole per liter for gulonolactone. Enzymatic hydrolysis was observed to be a first-order reaction, but non-enzymatic hydrolysis, a zero order reaction (Figs. 4–5).

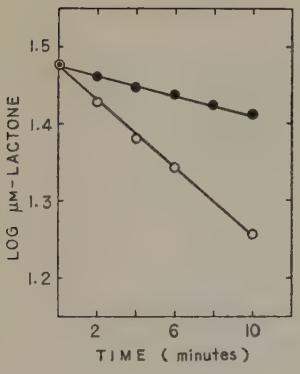

Fig. 4, a Effect of time on Lactonased-I
—●— Glucuronolactone
—○— Gulonolactone

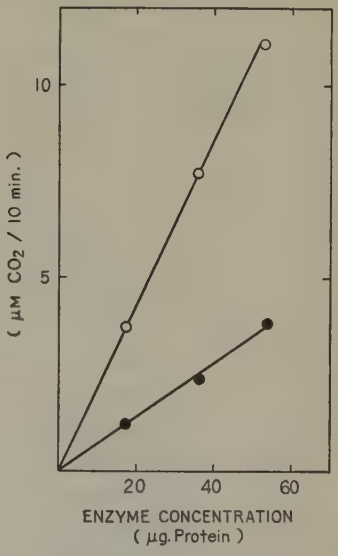

Fig. 4, b Effect of enzyme concentration on Lactonase-I
—○— Gulonolactone
—●— Glucuronolactone

Distribution in Various Organs—Activity of the enzyme was tested in various tissues of rat and it was found only in the liver, and not in the kidneys, brain, lung, spleen, and heart.

Intracellular Distribution of Lactonase-I and-II

As shown in Table V, there were activities on both glucurono- and gulonolactone in the soluble fraction, while there was only glucuronolactonase activity in the microsomal fraction. Lactonase activity was not detected in the mitochondrial fraction on either lactones.

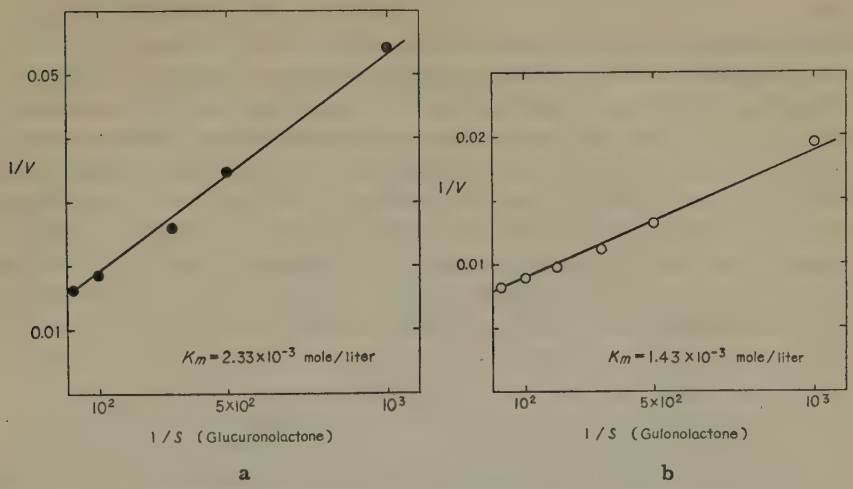

FIG. 5. Effect of substrate concentration on Lactonase-I
$1/V$—(μl. CO_2/2 min.)$^{-1}$; $1/S$—(l./M)

These data suggest the existence of two distinct lactonases, which, as mentioned in the Introduction, were tentatively named Lactonase-I and -II for soluble and microsomal fractions, respectively.

TABLE V

Intracellular Distribution of Lactonase

Cell fraction	Activity (*units/g-wet weight*)		Activity ratio Gul-L/Gl-L
	Glucurono-lactone	Gulono-lactone	
Mitochondria	0	0	
Microsome	11.8	0	
Soluble	90	297	3.3
Total activity	101.8	297	2.9

Conditions tested were the same as in Fig. 2, all activities are expressed as micromoles lactone hydrolyzed per equivalent to 1 g. of wet tissue during the initial 10 minutes. Microsomal suspension was added to reaction mixture immediately before the gas exchange, due to the alkalilability.

Differntiation of Lactonase-I and -II

Some differences between these two enzymes, Lactonase-I and -II, could be pointed out. First, the most striking was the fact that, as mentioned above, Lactonase-I was distributed definitely in the soluble fraction, and

Lactonase-II, in the microsomal fraction. Secondly, Lactonase-II was unstable to alkali treatment in 0.1 *M* sodium bicarbonate solution of pH 9.0 for 30–40 minutes. Another difference lay in the substrate specificity; Lactonase-II acted only on glucuronolactone and not on D-gulono-, L-gulono-, D-mannono-, D-manurono-, or L-gluconolactone, whereas Lactonase-I had a broad substrate specificity. In some cases, it was observed that the microsomal fraction showed activities on D-gulonolactone and other lactones, in addition to D-glucuronolactone. However, when these microsomal fractions were resuspended in 0.25 *M* sucrose and were retreated with supercentrifugation once or twice more, the activity on D-gulonolactone and others decreased or disappeared, although activity on D-glucuronolactone remained as before.

DISCUSSION

Presence of the activity of glucuronolactonase was first demonstrated by Eisenberg *et al.* (*10*) in the rat liver homogenate and slices. They identified the reaction product as D-glucuronic acid and stated that this activity disappeared at pH below 6.5. Further, Lehninger *et al.* (*3*) observed the presence of gulonolactonase activity, in addition to the glucuronolactonase activity, in the rat liver extract, and they said that these activitities were stimulated by Mg^{++}, Mn^{++}, or Co^{++} ions.

All of these results, however, were obtained using crude preparations. In the present work, purified lactonase was successfully obtained and the results concerning the effect of pH and metal requirements agree with those of Lehninger and Eisenberg, except the ineffectiveness of Co^{++} ion.

The activity ratio of gulonolactonase to glucuronolactonase (Gul-L/ Gl-L) was constant at about 3.4 at each step of purification, and the optimum pH for these two lactones was in the same range. Therefore, it is concluded that a single enzyme is responsible for the lactonase activities on these two substrates.

In the cell fraction, as can be seen in Table V, Lactonase-I showed the value of 3.3 for the ratio of Gul-L/Gl-L. On the other hand, the value of about 3.0 was obtained from the summed-up value of activities of Lactonase-I and -II, which were determined separately. As mentioned above, the ratio was always maintained at about 3.4 in each step of purification of the lactonase starting from acetone powder. Therefore, it may reasonably be concluded that Lactonase-II probably either loses its activity during the process of preparation of acetone powder or is not extracted from the acetone powder.

As shown in Table III, inhibition with heavy metal ions and SH-blocking reagents, and the reversal effect of glutathione or cysteine, indicate that Lactonase-I is an SH-enzyme. Michaelis constant of this enzyme was rather high with regard to glucuronolactone, as well as to gulonolactone. As glucono-δ-lactonase described by Brodie and Lipman (*22*) also showed a high Michaelis constant, it seems that lactonases in general have high Michaelis constant.

The glucono-δ-lactonase reported by Brodie and Lipman had optimum pH of 6.0, whereas the activity of Lactonase-I disappered completely at pH under about 6.5. Besides, there are differences between the glucono-δ-lactonase and Lactonase-I in the substrate specificity. Thus, these two enzymes are different from each other and there must be two enzymes showing gluconolactonase activity in the liver, namely Lactonase-I and the lactonase described by Lipmann.

As shown in this work, a differentiation between Lactonase-I and -II can be obtained from the differences in intracellular distribution, substrate specificity, and alkali-lability. Absence of gulonolactonase activity in the microsomal fraction is of interest. This finding suggests that if gulonolactone should be metabolized in microsome, it might follow another pathway. Burns (*23*) reported that rat liver microsome converted L-gulonolactone to L-ascorbic acid.

Examination of the distribution of Lactonase-I and-II in various animals in this laboratory (*11*) showed that Lactonase-I is found in the liver of rat, bovine, rabbit, and in the kidneys of pigeon, all of which are capable of synthesizing L-ascorbic acid. Human and monkey livers lacked the Lactonase-I, and the guinea pig liver had a different kind of lactonase, and all of these animals are known to require ascorbic acid from the diet. On the other hand, Lactonase-II was present in all the species tested. This grouping of animal tissues based on the presence of Lactonase-I is in reasonable agreement with the grouping by Lehninger *et al.* (*24*), which was based on the ability to biosynthesize ascorbic acid from gulonic acid. This suggests that Lactonase-I plays an important role in the biosynthesis of L-ascorbic acid. This subject was discussed in more detail in a previous report (*11*).

Physiological significance of Lactonase-II was not clarified by the present work and it requires futher investigantion.

As mentioned in the enzyme properties, the back reaction of the Lactonase-I was demonstrated to some extent with regard to D-glucuronic acid, but to a very small extent with L-gulonic acid. The lactone formation from gulonic acid might require some unknown factor or factors, or it might be achieved through some intermediates. Details of this lactonization is now under investigation.

SUMMARY

1. A presence of two types of lactonase in the cell fractions of animal tissues has been demonstrated. They were tentatively named Lactonase-I and -II for soluble and microsomal fraction, respectively.

2. Lactonase-I has been purified 21-fold from acetone powder. It required divalent cations such as Mg^{++} and Mn^{++} for activation. It has also been shown to have optimum pH 6.8–7.0.

3. Lactonase-I has been shown to have a rather broad substrate specificity and to act on L-gulonolactone, D-glucuronolactone and other lactones, whereas Lactonase-II was specific to glucuronolactone.

4. Lactonase-I has been proved to be an SH-enzyme. Michaelis

constant has been determined. Lactonase has been found in the liver of a rat but not in the kidneys, brain, lung, spleen, or heart.

5. The significance of this enzyme in the L-ascorbic acid biosynthesis has been discussed.

The writer's thanks are due to Prof. N. Shimazono and Dr. S. Ishikawa and the other members of this laboratory for their kind guidance and assistance, and to Dr. H. S. Isbell and Dr. T. Nakazima who kindly donated part of the substrates.

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CYANIDE INSENSITIVE TERMINAL RESPIRATORY SYSTEM IN AEROBACTER CLOACAE

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Many studies have been carried out on the terminal electron transport systems (terminal oxidase systems) in microorganisms. Most of these studies have confined that the main terminal oxidase system in microorganisms is cytochrome-cytochrome oxidase system as well as in mammalian tissues. Other terminal oxidase systems such as DPNH oxidase-peroxidase system (*1*) have also been reported, and many speculations have been done concerning the existence of the terminal oxidase system which does not possess cytochrome or cytochrome oxidase. However, it is quite unknown whether or not these terminal oxidase systems are coupled with the energy generating system such as the oxidative phosphorylation system, and the physiological significance of these systems is quite obscure.

On the other hand, as it is well known, microorganisms have an ability to exhibit characteristic biochemical phenomena concerning the adaptability to an environment, especially to produce an adaptive enzyme, which is quite unexpectable in animal and higher plant. It may be no exaggeration to say that microorganisms possess an ability to exhibit all possible sorts of fundamental biochemical phenomena. This conception may also be applicable in the bacterial respiratory system which is coupled to energy generating system.

As a first step to study this ability, several bacterial strains which can grow in a medium containing potassium cyanide, were isolated from soil. One of them, belonging to *Aerobacter cloacae*, showed a cyanide-insensitive growth and a respiration. In the present paper, studies were carried out concerning the nature of cyanide insensitive respiration in *Aerobacter cloacae* No. 26.

EXPERIMENTALS

Cultivation—Bacteria were aerobically grown in a bouillon medium (pH 7), containing meat extract 1 per cent, peptone 1 per cent, and NaCl 0.5 per cent in a one to one mixture of distilled water and tap water. The growth rate of bacteria were measured using nepherometer.

Measurement of Oxidative Activity—The oxidative activity for several substrates was estimated by measuring the rate of oxygen uptake using Warburg respirometer at 30°.

Estimation of Cyanide in Medium—Cyanic acid in a medium was absorbed in alkaline solution using Conway's microdiffusion apparatus (*2*) and was estimated by titrating with diluted solution of silver nitrate in the presence of potassium iodide as an indicator (*3*). Each Conway's unit contained 1 ml. of sample and 1 ml. of 6 *N* H_2SO_4 in an outer portion, and 1 ml. of 1 *N* NaOH in an inner portion. Usually, microdiffusion was carried out for 4 hours at 30°.

Detection of Hydrogen Peroxide—In the presence of hydrogen peroxide, iodine is formed from potassium iodide in acid solution. Then iodine is detected by iodine-starch reaction.

Spectrophotometric Analysis—Cytochrome components were detected spectrophotometrically based on the opal-glass method (*4*).

Materials—Crystalline alcohol dehydrogenase was kindly supplied by Dr. T. Kôno, Department of Agricultural Chemistry, University of Tokyo. DPN (90 per cent pure) was prepared by the method of Okunuki *et al* (*5*). DPNH was prepared by reducing DPN with alcohol dehydrogenase (*6*).

RESULTS

Isolation and Identification of Cyanide Insensitive Bacteria—Thirty strains of bacteria which could grow in a bouillon medium containing 10^{-3} *M* of potassium cyanide, were isolated from soil. Among them, strain No. 26, which could grow in cyanide-bouillon medium most rapidly, was found to belong to *Aerobacter cloacae* according to Bergey's Manual of Determinative Bacteriology (*7*).

Rod; 0.4–0.6 × 1.2–1.6 μ, active motile with peritrichous flagell, gram negative, endospore not formed.

Agar colony; circular, smoth, entire, to slightly erose in old culture, pale brown, opaque, butyrous.

Agar slant; abandant growth, filiform, dirty white, glistening, medium unchanged.

Nutrient broth; ring, strong fluid turbidity, flocculant sediment.

Nutrient gelatin; 20° liquefaction.

BCP milk test; acid coagulate.

Methyl red test; negative.

Voges Proskauer test; positive.

Nitrate; reduced to nitrite.

Indol; not formed.

Hydrogen sulfide; not formed.

Citrate; utilized.

Catalase; positive.

Fermentation;

	Glycerine	Xylose	Glucose	Sucrose	Lactose
Acid	+	+	+	+	+
Gas	±	+	+	+	−

Optimal temperature; 30°–37°. Facultative aerobic.

Then following experiments were carried out using *Aerobacter cloacae* No. 26.

Effect of Cyanide on Bacterial Growth—Fig. 1 shows the growth of *A. cloacae* in bouillon medium in the presence or absence of 10^{-3} *M* of cyanide, respectively. In the presence of cyanide, though the lag period was prolonged,

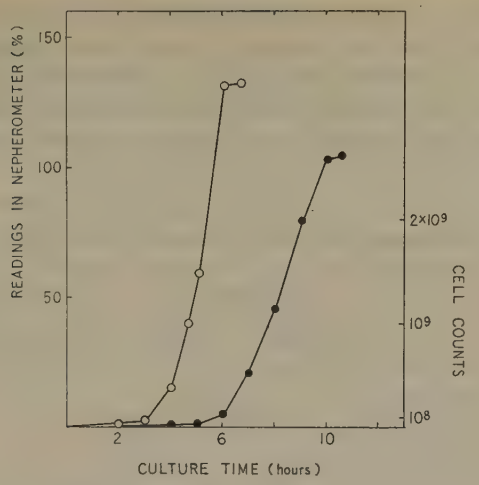


FIG. 1. Effect of cyanide on growth of *A. cloacae* in bouillon medium.

Inoculum size of cell was 10^6/ml. Cultivation was carried out on Monod's cultivation apparatus.

—○— without cyanide, —●— with 10^{-3} *M* cyanide.

significant growth inhibition was not observed at the logarithmic period. At the logarithmic period, almost all amount of cyanide remained still in the medium. This result shows that the bacteria have an ability to do cyanide-insensitive respiration adaptively.

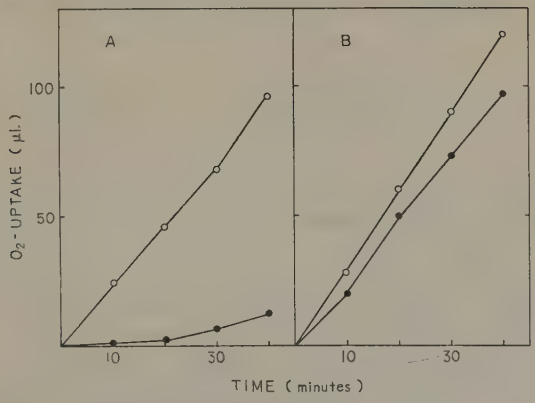


FIG. 2. Effect of cyanide on glucose oxidation.

Each Warburg vessel contains, 4×10^9 cells, 0.2 ml. of $10^{-2}M$ KCN and 1.6 ml. of *M*/15 phosphate buffer (pH 7.4) in main compartment, 0.2 ml. of 0.2 *M* glucose in side arm and 0.2 ml. of 20 per cent KOH in center well. (A): with cyanide non-adapted cells, (B): with cyanide adapted cells.

—○— without cyanide, —●— with cyanide.

Effect of Cyanide and Other Respiration Inhibitors on Glucose Oxidation by Resting Cells—Effect of cyanide on the oxidation of glucose are shown in Fig. 2. The respiration of cyanide non-adapted cells was remarkably inhibited by cyanide, whereas that of cyanide adapted cells was only slightly inhibited by cyanide. Between both cases, no significant difference was found in Q_{O_2} and stoichio-

TABLE I

Stoichiometry of Glucose Oxidation by Resting Cells

	Consumed (μM)		Formed (μM)	Q_{O_2}
	Glucose	Oxygen	Carbon dioxide	
Cyanide non-adapted cell	1.1	4.1	3.9	500
Cyanide adapted cell	1.1	4.6	4.5	600

Conditions were similar given in Fig. 2.

metry of glucose oxidation as shown in Table I. Carbon monooxide (95 per cent) and sodium azide (10^{-3} *M*) showed no effect on glucose oxidation in all cases.

Disappearance of Cyanide Insensitivity in Cyanide Insensitive Cells—When the cyanide insensitive cells were grown in cyanide free medium, the insensitivity to cyanide in the glucose oxidation was disappeared as shown in Fig. 3. It is of interest that the insensitivity to cyanide has still retained during first to third generation, followed by the sudden decrease during fifth to seventh

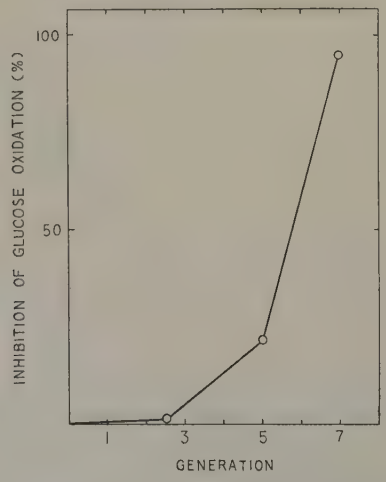


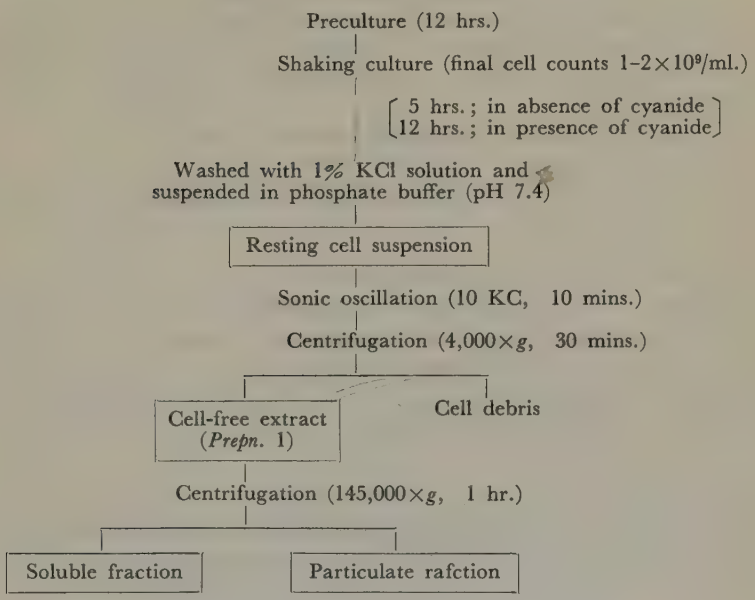
FIG. 3. Disappearance of cyanide insensitivity in cyanide insensitive cells. Conditons were the same given in Fig. 2.

generation. Then, after seventh division, cells showed a typical cyanide sensitive respiration.

Preparation of Cell-free Respiratory System—It is of important to determine whether the respiratory enzymes in glucose oxidation or the terminal oxidase system is altered during the adaptive formation of cyanide insensitive respiratory system. Therefore, further studies were carried out using cell-free extracts as an enzyme preparation. The method for the preparation of cell-free extracts are given in Scheme 1. The cell-free preparation (*Prepn. 1*) thus

SCHEME 1

Preparation of Cell-free Extract



obtained, possessed an oxidase activity towards the members of tricarboxylic acid cycle such as α-ketoglutarate, succinate and malate. The oxidase was remarkably activated by the addition of DPN, suggesting the existense of DPNH linked terminal oxidase system. The preparation also possessed catalase activity which is perfectly inhibited by cyanide (10^{-3} *M*).

Effect of Cyanide on the Oxidation of DPNH and Succinate—The effect of cyanide on the oxidation of DPNH and succinate by the extract (*Prepn. 1*) are shown in Table II. Cyanide showed no inhibitory action on the oxidation of both DPNH and succinate oxidizing system obtained from cyanide sensitive as well as cyanide insensitve cells. This fact indicates that the terminal oxidase system in *A. cloacae* No. 26 are cyanide insensitive in nature. Furthermore, the production of hydrogen peroxide was not detected during the oxidation of DPNH and succinate in the presence of cyanide, suggesting the imparticipation of flavoprotein as a terminal oxidase.

TABLE II

Effect of Cyanide on Oxidation of DPNH and Succinate by Cell-free Extract

Cyanide		Oxygen uptake in 20 minutes ($\mu l.$)		
		DPNH (5 μM)	DPNH*	Succinate (40 μM)
A	−	50	14.9	36.4
	+	52	15.8	36.2
B	−	58	21.0	27.0
	+	55	24.9	28.0

Each Warburg vessel contained, 0.5 ml. of cell-free extract, 0.7–1.3 ml. of phosphate buffer (pH 7.4), 0.2 ml. of 10^{-2} *M* KCN (or water) in main compartment, 0.2 ml. of substrate in side arm and 0.2 ml. of 20 per cent KOH in center well. Total volume 2.2 ml.

A; extracts prepared from cyanide non-adapted cells.

B; extracts prepared from cyanide adapted cells.

* DPNH was generated by reducing DPN with alcohol and alcohol dehydrogenase in vessel.

Localization of Terminal Oxidase Systems in Cell—As shown in Fig. 4, crude extracts (*Prepn. 1*) was centrifuged at 145,000×*g* for one hour. The oxidase activity of supernatant fluid and particulate fraction towards DPNH and succinate was shown in Table III. Though a larger part of oxidase activity was localized in particulate fraction in both cases, a detectable activity was also observed in the supernatant fluid. In the case of DPNH oxidation, the

TABLE III

Oxidation of DPNH and Succinate by Particulate and Soluble Fraction

	Oxygen uptake in 30 minutes ($\mu l.$)	
	DPNH*	Succinate
Cell-free extract (*Prepn. 1*)	16.5	33.5
Soluble fraction	2.8	7.0
Particulate fraction	10.2	32.8
Soluble fraction+ particulate fraction	19.2	33.0

Conditions were similar given in Table II.

* DPNH was generated by reducing DPN with alcohol and alcohol dehydrogenase in vessel.

activity of the mixed solution of particulate and soluble fractions was stronger than the sum of the activities of the particulate fraction and that of the soluble fraction. This fact indicates that the soluble fraction contains some important component for the DPNH oxidizing system in the particulate frac-

tion. However, any possible component such as cytochrome has never been detected in the soluble part.

Spectrophotometric Analysis—Using hand-microspectroscopic method, only a single absorption band was detectable near 560 mμ by the addition of dithionite. Fig. 4 shows the absorption spectra of the particulate fraction prepared from cyanide insensitive cells. Two peaks are observed near 530 and 557 mμ in all cases, showing the presense of cytochrome. The optical density at 557 mμ in the presence of DPNH, succinate and dithionite was larger than

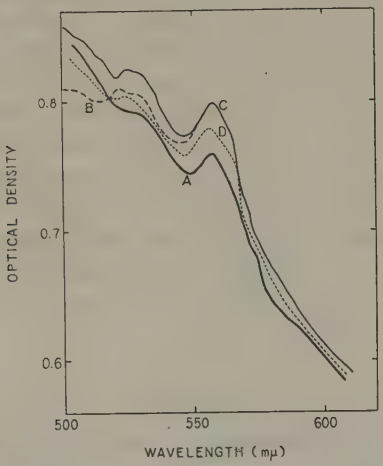


Fig. 4 Absorption spectra of particluate fraction. A; control, B; dithionite added, C; DPNH added, D; succinate added.

that of the control experiment, suggesting the participation of the cytochrome as a member of the terminal oxidase system. The figure of the spectrum did not change after the treatment with cholate, protease, or alkaline buffer (pH 8.9), and also unchanged in the presence of cyanide. A quite the same result was obtained in the case of the particulate preparation obtained from cyanide sensitive cells. Other cytochrome components were detected neither in the particulate nor in the soluble fraction. Nadi reaction (*8*) was also negative, suggesting the absence of cytochrome oxidase.

DISCUSSION

From the experiment with cell-free extracts, it may be evident that the terminal oxidase systems in *A. cloacae* are cyanide insensitive in nature. The facts that the respiratory system in this organism is not affected by the presence of carbon monooxide or sodium azide, and that the cytochrome component is quite the same in both cyanide sensitive and insensitive one, support the conclusion given above. However, it is doubtful whether the phenomena observed in the cell-free extracts may also occur in intact cells, *i.e.*; it is probable that the partial modification occurred in the terminal oxidase system

during the course of the extraction. Though the essential components of the terminal oxidase systems in *A. cloacae* are yet unknown, it seems probable that cytochrome 557 plays a significant role in the system. As the terminal oxidase system other than cytochrome oxidase system, DPNH oxidase-peroxidase system in *Streptococcus* (*1*) and the unknown system in *Poky* strain of *Neurospora* (*9*) and *petite* colony of yeast (*10*) are reported. In these systems, flavoprotein was thought to be an important component as a terminal oxidase. However, it is doubtful whether these terminal oxidase systems may be coupled with the energy generating system. In case of *A. cloacae*, the terminal oxidase seems not to be a flavoprotein because of the following findings; (a) participation of cytochrome 557 in terminal oxidase system, (b) non-production of hydrogen peroxide during the terminal oxidase reaction, (c) localization of the terminal oxidase system in particulate fraction. Furthermore, the growth of *A. cloacae* was cyanide insensitive as well as their respiration, and the Qo_2 was so large compared with that of the flavin respiration organisms. These results may suggest that *A. cloacae* possesses a cyanide insensitive terminal oxidase system which can be coupled with energy generating system. It remains still as an important problem to be clarified the essential difference between the respiratory system of cyanide sensitive cells and that of cyanide insensitive cells.

SUMMARY

1. *Aerobacter cloacae* isolated from soil was found to show a cyanide-insensitive growth adaptively.

2. The glucose oxidation by cyanide adapted cells was cyanide insensitive, whereas that by cyanide non-adapted cells was remarkably inhibited by cyanide.

3. The oxidation of DPNH and succinate by the cell-free extract prepared from both cyanide adapted and cyanide non-adapted cells, was cyanide insensitive. Almost all of the oxidase activity was localized in the particulate fraction.

4. A cytochrome component which has absorption maxima at 557 and 530 mμ was detected in particulate fraction and was found to be reduced by the addition of DPNH, succinate and dithionite.

5. The physiological significance of this cyanide insensitive terminal oxidase system was discussed.

The authors wish to express their sincere appreciation to Mr. K. Komagata, The Institute of Applied Microbiology, University of Tokyo, for his kind advise in the diagnosis of bacteria.

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ACTION OF TRYPSIN UPON SYNTHETIC POLY-ε-AMINOCAPROYL-α-ALANINES*

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The synthesis of several linear poly-ε-aminocaproyl-α-amino acids such as poly-ε-aminocaproyl-DL-alanine have recently been achieved by Noguchi and his coworkers with the aid of their "*N*-carbothiophenylamino acid method" (*1*). Among these synthetic polymers, poly-ε-aminocaproyl-DL-alanine (*2*), poly-ε-aminocaproyl-α-aminoisobutyric acid (*3*), and poly-ε-aminocaproyl-L-proline (*4*), the Bence-Jones protein like substance, possess an unusual property—they are reversibly coagulated by heat in acidic or neutral aqueous solution (*2–4*).

In the previous communication (*5*) the author briefly reported that these polymers can be partly hydrolysed by the action of trypsin if relatively high concentrations of the enzyme are employed. In the present paper the author wishes to report more extensive studies on this tryptic behavior and the effect of temperature on the enzymatic hydrolysis of poly-ε-aminocaproyl-α-alanines.

EXPERIMENTAL

Materials—Poly-ε-aminocaproyl-DL-alanine (molecular weight, 21,800 (*2*)) and poly-ε-aminocaproyl-L-alanine (molecular weight, 7,000) were prepared by polymerization of the respective *N*-carbothiophenyl derivatives.

ε-Carbothiophenylaminocaproyl-L-alanine methyl ester was obtained from ε-carbothiophnylaminocaproylchloride (*2*) and L-alanine methyl ester in ether solution as oily product in 94 per cent yield.

ε-Carbothiophenylaminocaproyl-L-alanine was synthesized as follows. ε-Carbothiophenylaminocaproyl-L-alanine methyl ester was hydrolyzed in a mixture of 2 *N* sulfuric acid and glacial acetic acid (equal volume) for 30 minutes at 100°. This compound was recrystallized from ethyl acetate; yield, 52 per cent; m.p., 71–72°; $[\alpha]_D^{18}$ −18.8° (absolute alcohol; c, 2.0 per cent).

Analysis Calcd. for $C_{16}H_{22}O_4N_2S$: N 8.23, COOH 13.29 per cent.
Found: N 8.26, COOH 13.82

Poly-ε-aminocaproyl-L-alanine was prepared by the polymerization of ε-carbothiophenylaminocaproyl-L-alanine. Polymerization of 5 g. of the carbothiophenyl compound for 26 hours

* Part of this work was presented at the Symposium on Protein Structure in Tokyo, November, 1955 and at the 9th Annual Meeting of the Chemical Society of Japan in Tyoto, April, 1956.

at 135° and then 2 hours at 150° gave a non-crystalline solid. The substance was purified with acetone and ether; yield, 98 per cent; $[\alpha]_D^{16}-11.3°$ (glacial acetic acid; c, 2.0 per cent). Molecular weight was estimated to be 7,000; mean grade of polymerization, about 38.

Analysis Calcd. for $H_2O(C_9H_{16}O_2N_2)_n$: N, 15.21 per cent ($n=\infty$)
Found: N, 15.11

Molecular weights of the polymers were estimated from osmotic pressure measurements and the N-terminal amino group titration values with perchloric acid. Denaturated ovalbumin was prepared by treating native ovalbumin at 100° and pH 6.0 for 15 minutes. Molecular weight of the original native ovalbumin prepared by the method of Sörensen and Höyrup (*6*) was estimated to be 35,000. The heat-coagulated ovalbumin was collected and washed with acetone, and dried *in vacuo*. Suspensions of the denatured protein were used as a reference substrate of trypsin.

Twice recrystallized trypsin (containing 50 per cent $MgSO_4 \cdot 7H_2O$ and 7.5 per cent protein nitrogen) purchased from the Nutritional Biochemicals Corporation (Trypsin I), and "Trypsilin" (containing 15.43 per cent protein nitrogen) obtained from the Mochida Pharma-

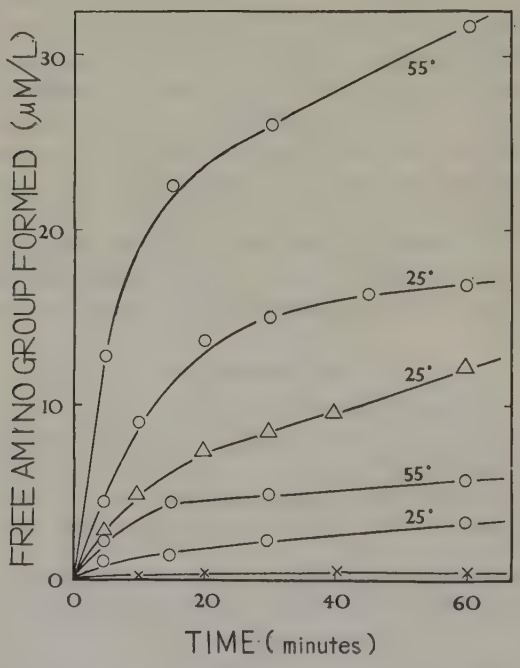

Fig. 1. Hydrolysis of poly-ε-aminocaproyl-α-alanines by trypsin. Reaction mixture contained 0.16 per cent the polymer in 0.013 *M* phosphate buffer solution (pH 7.1) in a final volume of 5.0 ml. ○ (higher curves): poly-ε-aminocaproyl-DL-alanine, 110 μg. of Trypsin II per ml. of the reaction mixture. ○ (lower curves): poly-ε-aminocaproyl-DL-alanine, 20 μg. of the enzyme per ml. Incubation temperature, 25° and 50°. △: poly-ε-aminocaproyl-L-alanine, 110 μg. of the enzyme per ml. Incubation temperature, 25°. ×: the enzyme free control test of all cases.

ceutical Mfg. Co., Ltd. (Trypsin II) were used as the enzyme. Their specific activities $\left([\text{T.U.}]^{\text{cas.}}_{\text{mg.P.N.}}\right)$ were 0.23 and 0.15 respectively. Crystalline α-chymotrypsin and carboxypeptidase were kindly supplied from Dr. N. Sakota of the Dainippon Zoki Laboratory.

Analytical Procedures—Hydrolysis of the polymers and the protein was followed by determining the formation of free amino groups by the ninhydrin method of Moore and Stein (*7*). To 4 ml. of 0.25 per cent polymers or ovalbumin solution was added 1 ml. of Trypsin I or II solution (in 0.013 *M* phosphate buffer solution, pH 7.1). In all cases enzyme-free and substrate-free controls were also run. The reaction was usually performed in an open test tube at 25° for 60 minutes. The reaction was stopped by freezing the reaction mixture in an ice-salt mixture, and 2.1 ml. of the ninhydrin reagent containing stannous chloride was added. After development of deep purple colour by heating, the mixture was diluted with the solvent, and the colour was measured by a photoelectric colorimeter at 575 mμ using L-leucine as the standard. Formol titration method (*8*) was also employed to determine the rate of hydrolysis, but no detectable difference was found between the results obtained by the two methods. Viscosity of the reaction mixtures was measured by an Ostwald viscosimeter.

RESULTS

Tryptic Cleavage of Poly-ε-aminocaproyl-α-alanines—As can be seen from Fig. 1, considerable hydrolysis of poly-ε-aminocaproyl-DL-alanine and poly-ε-aminocaproyl-L-alanine were observed in the presence of 110 μg. or 20 μg. per ml. of Trypsin II, as evidenced by the appearance of free amino groups. The amino groups did not appear when the concentrations of the enzyme were lowered to a level suitable for its usual protein substrates.

It seemed that relatively high concentrations of the enzyme were necessary

TABLE I

Decrease in Specific Viscosity of the Reaction Mixture

Time (*min.*)	Specific viscosity	
	0.43 per cent substrate*	0.60 per cent substrate**
0	0.046	0.25
10	0.041	0.20
20	0.035	0.19
30	—	0.18
40	0.034	—
60	—	0·17

* Reaction mixture contained 21.5 mg. of poly-ε-aminocaproyl-DL-alanine and 0.30 mg. of Trypsin I in a final volume of 5.0 ml. of 0.013 *M* phosphate buffer solution (pH 7.1). Incubation was carried out at 25°.

** Reaction mixture contained 30.0 mg. of the polymer and 0.60 mg. of Trypsin I in a final volume of 5.0 ml. of the phosphate buffer solution. Incubation temperature, 30°.

Change in the viscosity was measured at 30° with an Ostwald viscosimeter.

for the tryptic cleavage of the polymer, since only 1–10 μg. of the enzyme per ml. were required to give sufficient hydrolysis of denaturated hemoglobin, an usual substrate of trypin. The enzyme preparation retained its activity to capable of hydrolysing the polymers even after treatment with diluted hydrochloric acid (pH 2.2) for 24 hours at 25°. Under the optimal conditions the DL- and L-alanine polymers were hydrolysed to the extents of 0.2 and 0.4 per cent, respectively, in less than two hours.

It was possible to measure a change in viscosity of the reaction mixture during the hydrolysis, although the polymer solution has a rather low specific viscosity even in saturated solution. Typical results obtained with 0.43 and 0.63 per cent solutions of the polymer are shown in Table I.

Specific viscosity of the reaction mixture decreased slowly in the presence of trypsin. The decrease of the viscosity was not observed without the enzme.

Effect of pH on the tryptic hydrolysis of the polymers and denaturated ovalbumin was studied by measuring the rate of formation of free amino

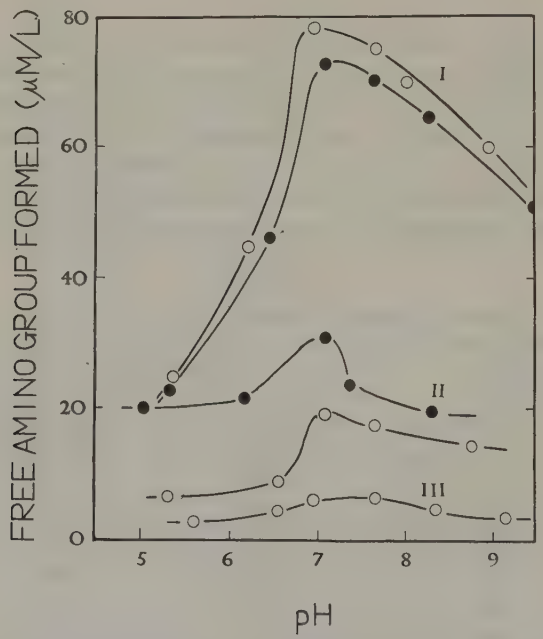

Fig. 2. Effect of pH on the hydrolysis of poly-ε-aminocaproyl-α-alanines and denaturated ovalbumin.

I: 0.2 per cent heat denaturated ovalbumin.

II: 0.2 per cent poly-ε-aminocaproyl-DL-alanine.

III: 0.2 per cent poly-ε-aminocaproyl-L-alanine.

○: Incubation at 30°, ●: at 60°. Each reaction mixture contained 0.2 per cent of substrate, 110 μg. per ml. of Trypsin II and 0.013 *M* phosphate buffer (pH 7.1) in a final volume of 5.0 ml. Incubation was carried out for 10 minutes.

TABLE II

Action of α-Chymotrypsin and Carboxypeptidase on Poly-ε-aminocaproyl-DL-alanine

Time (*min.*)	Free amino group formed (μM/liter)	
	α-Chymotrypsin*	Carboxypeptidase**
10	0.0	0.0
20	0.0	0.0
40	0.1	0.0
60	0.2	0.0

Experiments were carried out under the same conditon as described in Fig. 1 except for enzyme and its activator.

* Reaction mixture contained 0.50 mg. of crystalline α-chymotrypsin in a final volume of 5.0 ml.

** Reaction mixture contained 0.50 mg. of diisopropylfluorophosphate-treated carboxypeptidase aud 0.001 *M* $MgCl_2$ in a final volume of 5.0 ml.

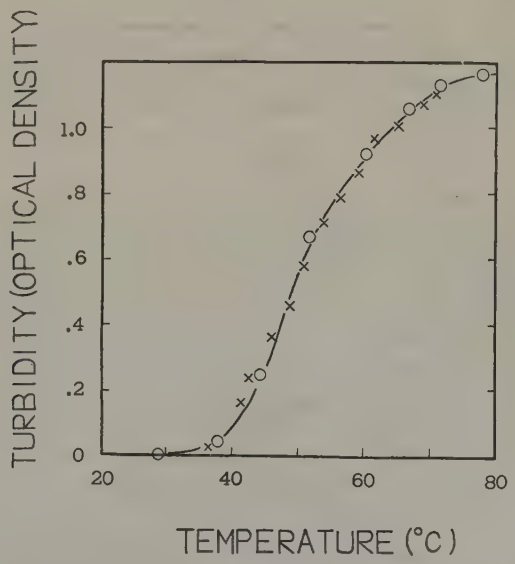

FIG. 3. Reversible heat coagulation of poly-ε-aminocaproyl-L-alanine solution.

○: points obtained with rising temperature.

×: points obtained with falling temperature.

Concentration of the poly-ε-aminocaproyl-L-alanine (mol. wt. 7,000), 0.24 per cent in 0.1 *M* formic acid (pH 2.3); thickness, 22.0 mm.; filter, 610 mμ; temperature, ±0.05°. Hirama electophotometer was used.

groups. Citrate buffer was used in the pH range from 5 to 7 and borax buffer for 7 to 10. As is illustrated in Fig. 2, the pH optima were found at pH 7.1 to 7.4.

These polymers made also resistance to the action of high concentrations of crystalline α-chymotrypsin and diisopropylfluorophosphate-treated carboxypeptidase under the same conditions as shown in Table II.

Effect of Temperature—It was found that on heating up to 40–70°, poly-ε-aminocaproyl-L-alanine precipitates from aqueous solution as the DL-alanine polymer does in like manner. The precipitate formed reversively goes back into solution on cooling. Fig. 3. shows the reversibility of the heat coagulation. This phenomenon suggests that some differences might exist between the structure of the precipitated form and that of the dissolved form. It seems therefore intresting to study the effect of temperature on the tryptic hydrolysis of the polymer and that of ovalbumin, a standard substrate.

As shown in Fig. 4, two maxima were observed in the temperature-hydrolysis curves of the DL-alanine and L-alanine polymers, due to their

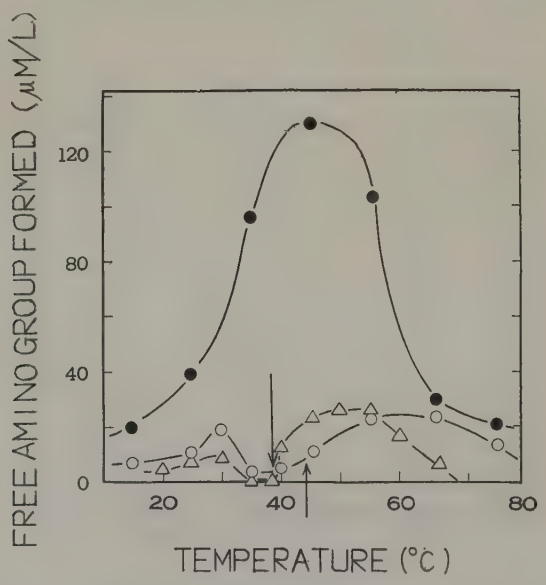

FIG. 4. Effect of temperature on the hydrolysis of poly-ε-aminocaproyl-α-alanines and denaturated ovalbumin.

Each reaction mixture consists of 0.2 per cent poly-ε-aminocaproyl-DL-alanine (○) or poly-ε-aminocaproyl-L-alanine (△) or heat denaturated ovalbumin (●), 110 μg. per ml. of Trypsin II and 0.013 *M* phosphate buffer solution (pH 7.1) in a final volume of 5.0 ml. Incubation was carried out for 10 minutes except the case of poly-ε-aminocaproyl-L-alanine (20 minutes incubation). ⟶: begining of heat coagulation.

properties to be coagulated by heating. The maxima oberved were at 30° and 60° for 10 minutes incubation or 30° and 50° for 20 minutes incubation. On the other hand, only one peak was found in the hydrolysis of denaturated ovalbumin under the same conditions. The polymers were completely soluble at 30°, whereas they were coagulated at 60° (ref. (*4*) and Fig. 3). When the temperature was raised from 45 to 55°, the initial rate of hydrolysis of the DL-alanine polymer increased two-fold, whereas that of the ovalbumin was lower at 45° than at 55° (Table III).

TABLE III

Effect of Temperature on the Hydrolysis of Poly-ε-aminocaproyl-DL-alanine and Denaturated Ovalbumin

Time (*min.*)	Free amino group formed (*μM/liter*)			
	Poly-ε-aminocaproyl-DL-alanine		Heat denaturated ovalbumin	
	45°	55°	45°	55°
0	0	0	0	0
5	5.6	13.0	99.2	70.4
15	10.2	22.6	231	123
30	22.3	26.0	—	—
60	27.0	31.8	248	142

Experiments were carried out under the same conditions as described in Fig. 4.

DISCUSSION

Information available by this time shows that only lysyl (*9–11*) and arginyl (*10*, *12*, *13*) peptide, it's amide or ester linkages are susceptible to the tryptic attack. The results reported here, however, clearly indicate that poly-ε-aminocaproyl-α-alanines can also be hydrolysed by the action of trypsin if relatively high concentrations of the enzyme are employed. It is interesting, in this connection, to note that the structure of ε-aminocaproic acid can be regarded as α-deaminolysine. In the polymer of ε-aminocaproyl-α-alanine the ε-amino group, except that of the N-terminal residue, is not in a free state. This is remarkable since the free amino groups of lysyl and arginyl residues have been believed to be required for the tryptic hydrolysis of the susceptible compounds. At any rate, the finding described above has an important bearing on the substrate specificity of trypsin and calls for a more extensive study on this point using a number of synthetic substrates.

It has been well known that denaturated protein is more easily hydrolysed than native protein by the action of proteases including trypsin. It was shown here that the coagulated form of the alanine polymer is more susceptible than the dissolved form (Fig. 4 and Table III). This interesting fact might be due to some probable difference in the structures of both forms and not to the difference of pH-optima (Fig. 2). Although the mechanism of the reversible

heat coagulation of the alanine polyers is not yet clear, it can be assumed that not only their solubility in water but other properties may also be altered by heating.

Both DL- and L-α-alanine polymers were susceptible to trypsin in either coagulated or dissolved form, but the extent of their hydrolysis was relatively small and no complete hydrolysis was observed. It is, however, an intrestin result that, in general, a polymer which has large molecular weight an contains only L-form of amino acid is easily hydrolysed rather than other one which has small molecular weight and contains D-form of amino acid. A possible mechanism of the tryptic cleavage of poly-ε-aminocaproyl-α-alanine will be shown and discussed in a later paper.

SUMMARY

Linear poly-ε-aminocaproyl-DL-alanine and poly-ε-aminocaproyl-L-alanine could be partly hydrolysed by the action of crystalline trypsin. Considerable decrease of specific viscosity of the reaction mixture occurred though total amounts of the hydrolysis were relatively small. Optimum concentrations of hydrogen ion in the reaction mixture were 7.1–7.4. These polymers make resistance to the action of high concentrations of α-chymotrypsin and carboxypeptidase. Two temperature optima were found at 30° and 60°.

The author wishes to express his hearty thanks to Prof. F. Egami of Nagoya University, J. Noguchi of this university, Dr. R. Sato, Osaka University and to Dr. N. Sakota, Dainippon Zoki Laboratory, for their kind encouragements throughout the course of this work. The author is also grateful to Miss K. Matsunaga for her technical assistances.

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CRYSTALLINE THIAMINE TRIPHOSPHATE; THE PREPARATION AND CHARACTERIZATION OF AUTHENTIC SPECIMEN

In view of the progress in biochemistry of various organic phosphorus compounds, our attention recently points to a possible physiological role of thiamine triphosphate (TTP), the presence of which was proposed in yeast cells (*1*) and also indicated in liver homogenate (*2*, *3*). In addition, H. Greiling and L. Kiesow (*4*) have suggested that TTP might serve an active transfer of phosphoric acid residues in cellular metabolism, just as already proved on ATP and its analogues.

Recently, A. Rossi-Fanelli *et al.* (*5*) have described on the preparation of crystalline specimen, although in minute amount, of TTP hydrochloride, with which they have failed in demonstrating the co-carboxylase activity. In their synthetic experiment, complete purification of this compound was so tedious and difficult, that its yield appeared to be exceedingly poor. Now, it seems to be of particular importance to establish a synthetic procedure suitable for the preparative purpose on one hand, and to clarify some undefined properties of TTP on the other, so as to settle the problems on biological significance of this compound.

The synthesis of this substance was done chiefly according to the prescription of M. Viscontini *et al.* (*6*). In the present experiment, it was found that the addition of phosphorus pentoxide to the reaction mixture could appreciably increase the yield of TTP. A mixture of 50 g. of thiamine hydrochloride and 50 g. of P_2O_5 was added in several portions into the melting phosphoric acid mixture at 100-105°, which was prepared from 130 g. of ortho-phosphoric acid (84 per cent) by preliminary heating at ca. 320°. The reaction was almost complete after standing at 100-105° for further 20 minutes under occasional stirring. After cooling, 80 ml. of distilled water were added and the resultant solution was centrifuged. The supernatant viscous liquid was slowly poured into 2 liters of cold ethanol-acetone mixture (1 : 1 in volume) under vigorous agitation. The white precipitate formed was collected, dissolved in minimal amount of water and precipitated as described above. After precipitations repeated thrice, the product was obtained as nearly nonhygroscopic white powder. Yield 63 g. After chromatographic analysis using resine column (IRC-50), this product was shown to be a mixture of the following components: TTP including higher phosphorylated thiamine (7 per cent), thiamine diphosphate (20 per cent), thiamine monophosphate (60 per cent) and free phosphate (below 1 per cent).

8 g. portion of the above product was dissolved in 10 ml. of water and chromatographed by means of IRC-50(H-form)-column (5.5×83 cm.) as

described by S i l i p l a n d i and S i l i p l a n d i (*7*). Development was effected by washing with dist. water at a flow rate of 50 ml./hour. Effluent fractions showing pH 2.8 to 4.0 were collected (*ca.* 400 ml.), and concentrated *in vacuo* up to *ca.* 10 ml. Two aliquots were combined (20 ml. of concentrate) and chromatographed again using somewhat smaller column (2.5×38 cm.). The rate of flow was adjusted to 10 ml./hour. Fractions of pH-range 3.0 to 3.8 were collected (*ca.* 20 ml.) and lyophilized into a snow-white powder, which showed a single spot unequivocally on the ascending paper chromatogram (R_f=0.13) irrigated with *n*-propanol/water/1 *M* formate buffer (pH 5.0)= 65 : 20 : 15 at 10°. Yield 504 mg. (3.15 per cent of the crude phosphate mixture used).

For further purification, 500 mg. of the above substance was dissolved in 3 ml. of water, and 2.3 ml. of absolute ethanol was slowly added so as to form moderate turbidity in the solution. On standing for several hours in

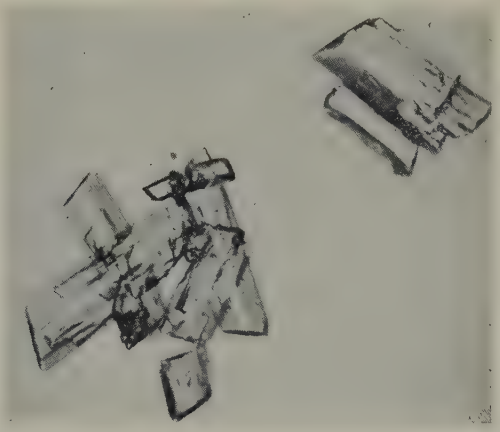

FIG. 1. Thiamine triphosphate from water (× *ca.* 200).

an ice-box (10°) the substance commenced to separate completely in prismatic crystals (Fig. 1).

After recrystallizations repeated twice, 346 mg. of pure crystals were recovered, which correspond to 2.2 per cent of the original mixture used. m.p. 194-197° (decomp.). (Found on air dried specimen : C 26.82, H 4.53, N 10.33, P 17.50. Calcd. for $C_{12}H_{20}O_{10}N\,SP_3 \cdot 2H_2O$: C 26.63, H 4.43, N 10.34, P 17.17). The substance is quite free from hydrochloric acid. It is easily soluble in water, but not in usual organic solvents. In aqueous solution, it decomposes gradually to form mono -(TMP) and di-esters (TDP) ; the reaction seems to be accelerated on heating. The absorption peak measured on an acidic solution (pH 4.4) containing 2 mg. of substance in 100 ml. of water was found at 242 mμ, whereas, after adjustment of pH with dilute sodium hydroxide, it was found at 234 mμ at pH 7, and 233 and 267 mμ at pH 9, respectively. For paper chromatography, the efficient separation was brought about by irrigation with *n*-propanol/water/1 *M* formate buffer (pH 5.0)=

65 : 20 : 15 on Tôyô No. 50 filter paper preliminary treated with 8-hydroxyquinoline by ascending method (10°, 17 hours) giving clear-cut blue-fluorescent spots as follows: R_f=0.13 for TTP, 0.20 for TDP, and 0.30 for TMP.

The detailed accounts will be published elsewhere.

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D-RIBOSE AS A SIMPLE RESPIRATION STIMULATING SUBSTANCE ON MURINE LEPROSY BACILLI WITHOUT REQUIRING ANY COFACTOR

Up to the present time, not a substance has been found to have a respiration stimulating effect on murine leprosy bacilli without requiring cofactors. Gray (*1*) observed that some fifty substrates or cofactors, including intermediates of glycolysis, members of the Krebs cycle, amino acids, miscellaneous compounds and coenzymes failed to stimulate the respiration during the 4 to 5 hours test period. Itō *et al.* (*2*) observed also that seventeen substrates, such as glycerol, succinate and vitamin B_{12}, did not stimulate the bacillary respiration, and that the respiration was stimulated only when heated liver or testicle extract of rats was combined with succinate.

In the course of metabolic studies of the substances delivered from diphosphopyridine nucleotide by diphosphopyridine nucleotidase of murine leprosy bacilli (*3*), this author found D-ribose, for the first time, to have a significant respiration stimulating effect on the bacilli without requiring any cofactor.

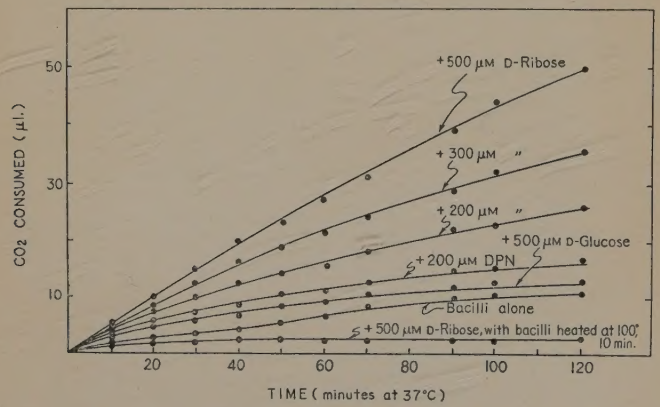

FIG. 1. Respiration stimulating effect of D-ribose on murine leprosy bacilli. Contents of the flask (total volume, 2.7 ml.): 10 mg. dry weight bacillary suspension, 200 μM phosphate buffer (pH 7.4), the substrate as indicated in Fig. and 0.2 ml. KOH (20 per cent) in center well.

Bacilli were collected purely from the rat lepromas by the method described in the previous paper (*4*), and their respiration was determined with a Warburg manometer in air.

Results shown in Fig. 1 revealed the stimulating effect of D-ribose on the bacillary respiration. This stimulation was enhanced with the increase in the concentration of D-ribose (from 200 to 500 μM).

Studies are undergoing to elucidate whether or not the respiration stimulating effect of D-ribose signifies directly the possession of metabolic pathway by the bacilli against D-ribose under aerobic condition. At any rate, however, the difference in the value of respiration between intact and heat-killed bacilli (Fig. 1) must indicate some participation of enzymatic activity of the bacilli in the respiration stimulating effect of D-ribose.

In conclusion, D-ribose was found, for the first time, to have a respiration stimulating effect on murine leprosy bacilli without requiring any cofactor.

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